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CHRONIC ADRENOCORTICAL DYSFUNCTION, INCLUDING ALDOSTERONE DEFICIENCY: STUDIES OF STEROID AND ELECTROLYTE METABOLISM*

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ABSTRACT

Metabolic studies were made on a 44-year-old man in whom acute adrenal insufficiency with vascular collapse and characteristic changes in electrolytes following a minor operation called attention to an unusual instance of adrenal dysfunction of at least twenty years' duration. Excretion of aldosterone was less than 0.5 microgram per twenty-four hours and was not augmented by restriction of sodium or administration of corticotropin or corticosterone. Loss of sodium and retention of potassium occurred with hyponatremia and hyperpotassemia during a period when the daily sodium intake was less than 200 mEq. Normal values for electrolytes were restored by administration of aldosterone or 9α-fluorocortisol, but not by use of corticotropin or cortisone. Values of plasma and urinary corticosteroids were normal but were not increased by corticotropin. Hydrocortisone and cortisone were identified in the urine. Results of glucose-tolerance tests were normal. Diuresis after ingestion of water was impaired. Urinary excretion of 17-ketosteroids was in the high normal range, and that of pregnanetriol and estrogens was moderately increased; the values for these constituents were not increased by corticotropin but were decreased by cortisone or corticosterone.

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THE occurrence of a state of adrenocortical insufficiency following a minor surgical procedure was found, on further study of the patient involved, to represent an unusual form of adrenocortical dysfunction. In the case to be reported, conspicuous features were aldosterone deficiency, unresponsiveness to corticotropin, normal levels of plasma and urinary corticosteroids, high-normal values for urinary 17-ketosteroids and increased excretion of pregnanetriol and estrogens.

Somewhat similar, but usually less complicated, alterations of adrenal function have been described by other investigators. Among these, Hudson and associates (1) and Pedersen and Sondergaard (2) demonstrated aldosterone deficiency without other abnormalities of adrenocortical function. Essentially the same situation was present in a case reported by Lichtwitz and co-workers (3), although the evidence for aldosterone deficiency was circumstantial. In the cases of a number of authors (4–12), some laboratory evidence for residual adrenal function was found despite clinical evidences of Addison's disease. The patient reported by Mandelstam's group (13) recovered clinically from acute adrenocortical insufficiency but some laboratory criteria of abnormal adrenal function remained.

None of these authors found laboratory evidence of adrenocortical hyperfunction associated with the hypofunction. Such a situation is known to occur in the syndrome of adrenal hyperplasia with virilization and a salt-losing tendency. In this syndrome, excessive excretion of pregnanetriol (14, 15) in addition to high excretion of 17-ketosteroids is associated with a lack of aldosterone, according to recent studies (16), in contrast to a high or normal excretion of aldosterone, as suggested by earlier work (17). Adult males with "virilizing" adrenal hyperplasia not associated with aldosterone deficiency and its metabolic consequences have been described by Prader and co-workers (17), Hedinger (18) and Perloff and Hadd (19).

REPORT OF CASE

A 43-year-old man came to the Mayo Clinic in July 1956, for his fifth examination. He had considered himself to be in good general health except for minor complaints of mild headaches, nasal obstruction and mild polydipsia, polyuria and nocturia. Deviation of the nasal septum appeared to be the only significant physical finding, and routine laboratory studies revealed no abnormalities.

Following submucous resection under topical anesthesia with cocaine, the patient complained of vertigo, nausea and vomiting, which became progressively more severe. Syncope when he got out of bed on the seventh postoperative day drew attention to the presence of postural hypotension. The presence of adrenal insufficiency was suspected, and determinations of serum electrolytes supported this impression. The values in milliequivalents per liter were as follows: sodium, 98; potassium, 6.2; chloride, 70; and carbon dioxide, 20. The blood urea level was 50 mg. per 100 ml.

He improved soon after administration of cortisone, 9α-fluorocortisol and an isotonic solution of sodium chloride. Steroid therapy then was discontinued and diagnostic

tests were made. Values for plasma cortisol and urinary formaldehydogenic corticosteroids were normal. Urinary 17-ketosteroids were 23 mg. per twenty-four hours, the normal range for men being 6 to 21 mg. The response to the "water test" was abnormal. No significant increase in plasma or urinary steroids was evident during a three-day period during which 25 units of corticotropin (ACTH) was given intravenously for eight hours each day. Roentgenograms showed no evidence of adrenal displacement or calcification. Values for pituitary gonadotropins, basal metabolic rate, serum protein-bound iodine, thyroidal uptake of I^{131}, and serum calcium and phosphate were all normal. The sperm count was 132,000,000 per ml.; motility and morphologic appearance were normal.

Since results of laboratory studies were not diagnostically decisive, search was made for relevant history and such physical findings as might be compatible with Addison's disease but that previously might have been overlooked. The familial and developmental histories revealed no abnormalities. The patient described a craving for salt, as well as mild polyuria (3 to 5 liters daily) and polydipsia, for as long as he could remember. These symptoms were associated with a high intake of salt and fluid. (He was later observed to flavor his food, of his own volition, with as much as 30 Gm. of salt daily.) After incision and drainage of a rectal abscess in 1937 and following hemorrhoidectomy in 1944, he had experienced nausea, vomiting and symptoms of postural hypotension, with spontaneous recovery each time. Diffuse melanosis with black freckles was present about the nape of the neck and on the forearms and hands. The palmar creases were melanotic, but no oral or scar or pressure-point pigmentation was evident. He was unaware of any change in cutaneous coloration. His weight and blood pressure, as recorded in his clinic record, had remained normal throughout the previous twelve years. Persistently low urinary specific gravity had been the only previously detected abnormal laboratory finding.

The studies to be reported were carried out in August and September 1956, and in February, March and April in both 1957 and 1958. Between periods of study, the patient received 0.1 mg. of 9α-fluorocortisol daily and 10 mg. of cortisone every eight hours. When last heard from in the spring of 1959, he was well and active.

METHODS

The values for serum sodium and potassium were determined by flame photometry. Urinary 17-ketosteroids were determined by the method of Callow, Callow and Emmens, and the values were corrected by the equation of Talbot and associates (20). A modification of the procedure of Corcoran and Page (21) was used for formaldehydogenic steroids. The method of Bongiovanni and Clayton (22) was used for pregnanetriol. Urinary estrogens were measured by bio-assay. Plasma cortisol was determined by a modification (23) of the Silber-Porter method.

For fractionation and isolation of 17-ketosteroids, the urine (1800 ml.) was extracted with four 225-ml. portions of butanol, the pH of the urine was adjusted to about 2, and again the urine was extracted with four 225-ml. portions of butanol. The butanol extract was washed with water, neutralized with a 10 per cent solution of sodium hydroxide and dried in a vacuum. Half of the residue was subjected to the hydrolytic procedure of Gallagher and co-workers (24). First, it was treated with β-glucuronidase (with a few milliliters of carbon tetrachloride as a preservative) for five days; it was extracted with chloroform and then was continuously extracted with ether at pH 1 for forty-eight hours; finally, the aqueous solution was boiled for ten minutes with a tenth volume of concentrated hydrochloric acid and extracted with chloroform. The 17-ketosteroids (16.1 mg.) were separated from more polar material by adsorption on a column of 40

Gm. of Florisil in methylene dichloride and elution with methylene dichloride that contained 4 per cent of methanol. The eluate then was chromatographed by the procedure of Lakshmanan and Lieberman (25). The individual 17-ketosteroids were purified further by chromatography on paper in Zaffaroni-type (26) solvent systems, crystallized and identified by means of their infrared spectra.

Determinations of tetrahydrocortisone (3α,17α,21-trihydroxypregnane-11,20-dione) were performed by the procedure that Eberlein (27) used on the urine of patients with adrenal hyperplasia, namely, hydrolysis with β-glucuronidase, extraction with methylene dichloride, chromatography on paper in his system[1] NE10 and quantitation by means of the Porter-Silber reagent.

Aldosterone was determined by a modification of the procedure of Neher and Wettstein (28); this modification by V. R. Mattox and M. L. Lewbart appears separately in this issue of the Journal. Determinations of cortisol and cortisone were made on the same extract. Chromatography of the extracts on paper in formamide-chloroform separated cortisol from cortisone and aldosterone. The cortisol then was chromatographed in system E_2B of Eberlein and Bongiovanni (29) and finally in system C of Bush. Quantitation was achieved by a method analogous to that of Neher and Wettstein (28) for aldosterone. The cortisone and aldosterone were separated by chromatography in formamide-butyl acetate-water (30) and quantitated in the same manner as cortisol after they had been chromatographed in system C of Bush.

Balance studies conducted in a metabolic ward under careful supervision included observations on the intake and output, in urine and feces, of nitrogen, sodium, potassium and chloride. Urine also was analyzed daily for creatinine and, at selected intervals, for 17-ketosteroids, formaldehydogenic steroids and aldosterone. Body weight and volumes of intake and output of fluids were determined daily. Values for serum electrolytes and the percentage volume of packed cells (hematocrit) were obtained on the first and fourth days of each six-day period. On these days, the plasma cortisol level was determined at 8 a.m., 11 a.m. and 4 p.m. The blood pressure was measured at least four times daily.

RESULTS

Electrolytes. Without steroid therapy, the patient was unable to retain sodium when given a diet including 89 to 100 mEq. of sodium (Table 1). Oral administration of 1 mg. of 9α-fluorocortisol or intramuscular injection of 200 and 400 μg. of aldosterone,[2] given in divided doses daily, caused sodium retention and potassium excretion. Data in Table 1, as well as further studies of renal function (Table 2), indicated that the salt-losing tendency was not caused by failure of the kidney to respond to salt-retaining hormone, as is the case in salt-losing nephritis. No abnormal urinary constituents were detected on repeated urinalysis, and retention of urea was observed only when postoperative stress occurred or sodium restriction was imposed.

Pertinent data of the metabolic balance studies are shown in Figure 1.

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[1] Composed of benzene (1000), t-butyl alcohol (100), methyl alcohol (400) and water (600).

[2] Natural aldosterone (100 μg. per ml.) in sesame oil.

TABLE 1. EFFECT OF STEROIDS ON EXCRETION OF ELECTROLYTES IN A CASE OF CHRONIC ADRENOCORTICAL DYSFUNCTION

No. of days	Electrolyte intake (mEq./day)		Medication	Urinary electrolyte excretion (mEq./day)	
	Na	K		Na	K
5	89	65	None	191	63
1	100	62	9α-Fluorocortisol, 1000 μg., orally	64	97
3	100	62	None	151	80
4	100	62	Aldosterone, 50 μg., i.m., q. 6 hrs.	101	106
2	100	62	Aldosterone, 100 μg., i.m., q. 6 hrs.	94	90
4	100	62	None	166	77

The charting in this figure is conventional, a negative balance being indicated by extension of the column above the zero line, and a positive balance by a clear zone below the zero line. The cross-hatched zones represent fecal excretion of electrolytes and nitrogen. Corresponding data on serum electrolytes, hematocrit, blood urea and body weight are shown in Figure 2.

An increase in sodium intake caused retention of sodium and loss of potassium. Reduction of sodium intake led to a net loss of sodium. Administration of ACTH and cortisone did not cause striking alterations in electrolyte balance. However, large doses of corticosterone brought about retention of sodium and loss of potassium. The cause of the decline in the

TABLE 2. SURVEY OF RENAL FUNCTION IN A CASE OF CHRONIC ADRENOCORTICAL DYSFUNCTION

Test	Results	Comment
Routine urinalysis	Sp. gr. 1.003 to 1.012; no abnormal constituents	No change since 1944
Maximal concentration of urine	Sp. gr. 1.015	Water deprivation, 14 hrs.
Response to antidiuretic hormone	Sp. gr. 1.022	Six hrs. after 10 units of vasopressin (Pitressin); water deprivation, 20 hrs.
Urea clearance	58 ml.	Urinary flow, 1.3 ml./min.; urea, 30 mg./100 ml.

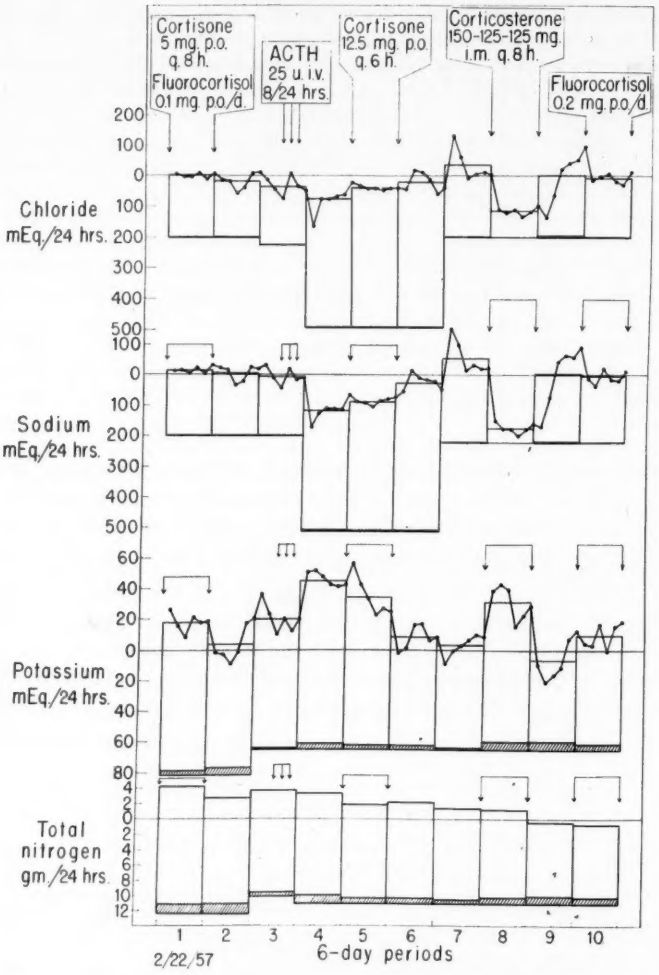
Cortisone 5 mg. p.o. q. 8 h.
Fluorocortisol 0.1 mg. p.o./d.
ACTH 25 u. i.v. 8/24 hrs.
Cortisone 12.5 mg. p.o. q. 6 h.
Corticosterone 150-125-125 mg. i.m. q. 8 h.
Fluorocortisol 0.2 mg. p.o./d.
Chloride mEq./24 hrs.
Sodium mEq./24 hrs.
Potassium mEq./24 hrs.
Total nitrogen gm./24 hrs.
2/22/57
6-day periods

FIG. 1. Effect of various steroids and ACTH on the metabolic balances of chloride, sodium, potassium and nitrogen in a case of chronic adrenocortical dysfunction. See text for details of charting.

concentration of serum sodium (Fig. 2) during the first three periods, without a corresponding net loss of sodium (Fig. 1), is not clear. Likewise, the continuing loss of weight over the first seven periods is not readily explained by the data. Perhaps a high intake of sodium prior to the study and subsequent losses of fluid as well as the persistent negative nitrogen balance help to explain the loss of weight.

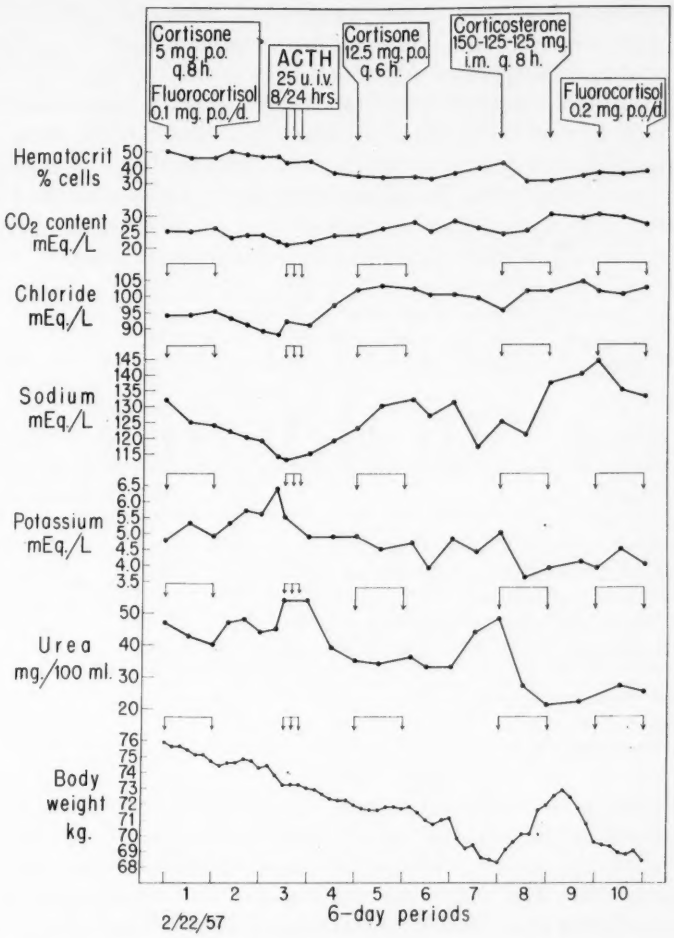

FIG. 2. Effect of various steroids and ACTH on serum electrolytes, hematocrit, blood urea and body weight in a case of chronic adrenocortical dysfunction.

Urinary aldosterone. A series of determinations of urinary aldosterone was made to determine whether the sodium-losing tendency was related to low adrenal output of this hormone (Table 3). Under conditions of the first 5 entries in this table, while dietary potassium was kept uniform at 68 mEq. per day, less than 0.5 μg. of aldosterone was found in each 24-hour specimen. Our normal range for adults with no restrictions on the intake of salt is 2 to 16 μg. per twenty-four hours. It is unlikely that these low levels in the urine of this patient were on the basis of unusual metabolism

of aldosterone in the body, since 11 and 18 μg. were the values found for urinary aldosterone when he received intramuscular injections of 200 and 300 μg. of aldosterone per twenty-four hours.

To measure the sensitivity of the assay, recovery experiments were carried out. A 48-hour specimen of urine was divided into equal parts. No aldosterone was found in one 24-hour aliquot. From the aliquot to which 1 μg. of aldosterone was added, 0.75 μg. was recovered. In a second experiment, a 24-hour aliquot contained 0.3 μg. of aldosterone and the portion to which 1 μg. of aldosterone was added contained 1.2 μg.

TABLE 3. URINARY EXCRETION OF ALDOSTERONE IN A CASE OF CHRONIC ADRENOCORTICAL DYSFUNCTION

Conditions	Aldosterone (μg./24 hrs.)	No. of specimens assayed
High-salt intake	<0.5	3
Low-salt intake	<0.5	7
Low-salt intake plus ACTH infusion	<0.5	3
Low-salt intake plus corticosterone, 400 mg./24 hrs.*	<0.5	2
High-salt intake plus cortisone, 50 mg./24 hrs.†	<0.5	1
Low-salt intake plus aldosterone, 200 μg./24 hrs.‡	11, 18	2
Low-salt intake plus aldosterone, 300 μg./24 hrs.‡	18	1

* Intramuscularly q. 8 hrs.
† Orally q. 6 hrs.
‡ Intramuscularly q. 8 hrs.

Carbohydrate metabolism. There was no clinical evidence of disturbed carbohydrate regulation. The blood sugar level with the patient fasting was consistently normal, even in the initial episode of acute adrenal insufficiency, when the value was 103 mg. per 100 ml. Results of 2 oral glucose-tolerance tests, which were performed while the patient was not receiving steroids, were abnormal only in that the two-hour values were 135 and 139 mg. per 100 ml., respectively.

Water metabolism. A deficiency in the regulation of water diuresis was evident from the results of the Robinson-Power-Kepler water test. The urea-chloride index was 2.7 initially (20 or higher is normal). When the test was repeated while the patient was receiving both cortisone and 9α-fluorocortisol, the urea-chloride index was 4 on one occasion and 16 on another.

Plasma and urinary corticosteroids. During the balance study, values for plasma cortisol and urinary formaldehydogenic corticosteroids were in the normal range but were unaffected by infusion of ACTH (Fig. 3).

The presence of cortisol (6 to 10 μg. per twenty-four hours) and cortisone (60 to 130 μg. per twenty-four hours) was established in several different 24-hour specimens of urine by chromatographic fractionation. This demonstrated at least a limited ability to oxygenate steroids at carbon atoms 11, 17 and 21.

On March 17 and 18, 1958, the patient excreted 2.5 and 2.3 mg. of tetrahydrocortisone per twenty-four hours (1.4 and 1.2 mg. per square

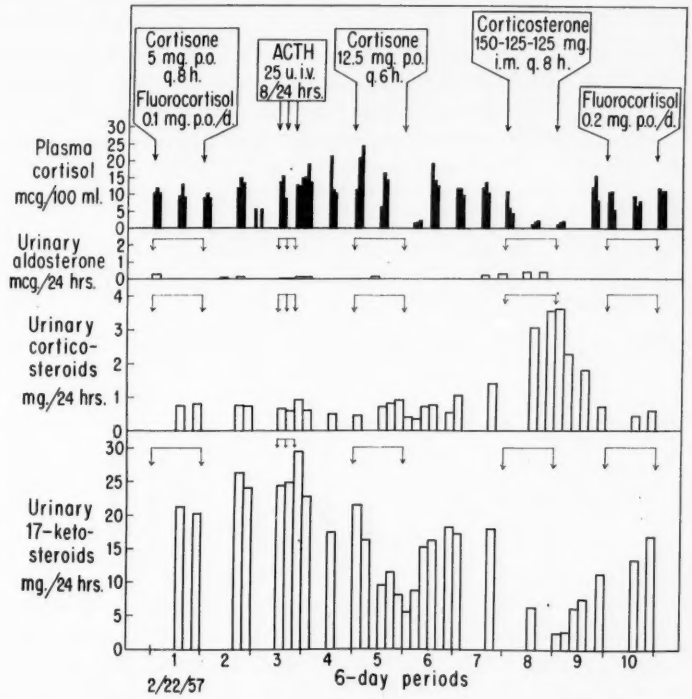
Cortisone 5 mg. p.o. q.8 h.
Fluorocortisol 0.1 mg. p.o./d.
ACTH 25 u. i.v. 8/24 hrs.
Cortisone 12.5 mg. p.o. q.6 h.
Corticosterone 150-125-125 mg. i.m. q. 8 h.
Fluorocortisol 0.2 mg. p.o./d.
Plasma cortisol mcg./100 ml.
Urinary aldosterone mcg./24 hrs.
Urinary cortico-steroids mg./24 hrs.
Urinary 17-keto-steroids mg./24 hrs.
2/22/57
6-day periods

FIG. 3. Effect of various steroids and ACTH on plasma cortisol level and on urinary aldosterone, corticosteroids and 17-ketosteroids in a case of chronic adrenocortical dysfunction.

meter of body surface per twenty-four hours). These values may be compared with the range of 0.6 to 2.4 mg. per square meter per twenty-four hours found by Eberlein (27) for 9 normal persons ranging in age from 6 days to 18 years. Our patient excreted less than 0.2 mg. of 3α,17α,21-trihydroxypregnane-20-one per twenty-four hours.

Urinary 17-ketosteroids. The average of 6 determinations of urinary 17-ketosteroids was 25 mg. per twenty-four hours when the patient was taking

neither steroids nor ACTH. After fractionation, the following substances were identified: isoandrosterone, androsterone, etiocholanolone, 11-keto-etiocholanolone and 11β-hydroxyetiocholanolone. The exact quantities of each of the pure substances were not determined; however, from the shape of the elution curve it appeared that the proportion of each of the 17-ketosteroids identified was within the range of variation found for normal persons. Excretion of 17-ketosteroids was not appreciably altered by infusion of ACTH but was suppressed by cortisone and corticosterone therapy (Fig. 3).

Urinary pregnanetriol. The patient excreted pregnanetriol in quantities four to six times the upper limit of normal (about 2 mg. in twenty-four hours) as found by Bongiovanni and Clayton (22) (Fig. 4). The excretion did not increase in response to exogenous ACTH given intramuscularly for six days.

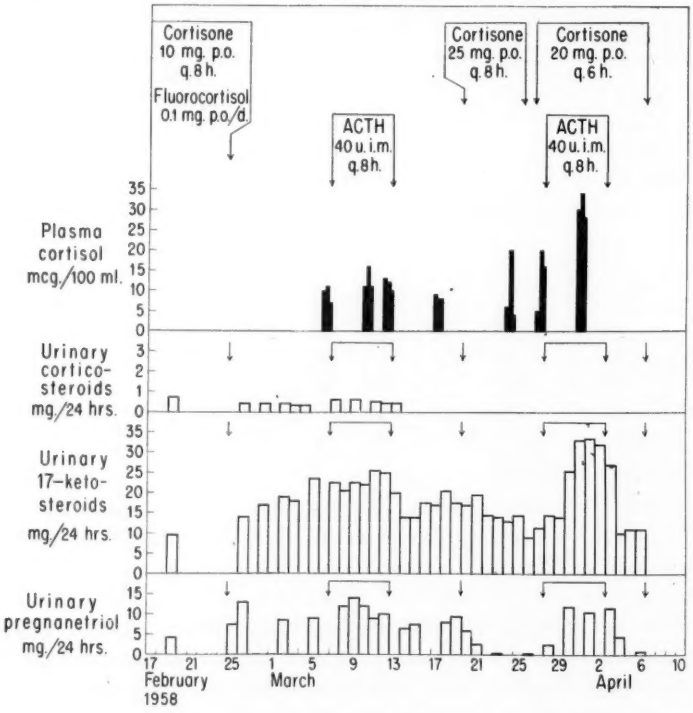
Cortisone 10 mg. p.o. q.8h.
Fluorocortisol 0.1 mg. p.o./d.
ACTH 40 u. i.m. q.8h.
Cortisone 25 mg. p.o. q.8h.
Cortisone 20 mg. p.o. q.6h.
ACTH 40 u. i.m. q.8h.
Plasma cortisol mcg./100 ml.
Urinary cortico-steroids mg./24 hrs.
Urinary 17-keto-steroids mg./24 hrs.
Urinary pregnanetriol mg./24 hrs.
17 21 25 1 5 9 13 17 21 25 29 2 6 10
February 1958
March
April

FIG. 4. Effect of various steroids and ACTH on plasma cortisol level and on urinary corticosteroids, 17-ketosteroids and pregnanetriol in a case of chronic adrenocortical dysfunction.

Responsiveness to ACTH. ACTH[3] failed to increase the levels of plasma and urinary steroids, suggesting that the adrenals already were functioning at their maximal capacity. Further evidence of this was obtained by suppressing the adrenals with cortisone and then administering ACTH while the use of cortisone was continued. Urinary 17-ketosteroids and pregnanetriol increased in response to this maneuver (Fig. 4). However, the previously observed limit of secretory capacity again was not exceeded, when one takes into account the probable contribution of exogenous cortisone to plasma cortisol and to urinary 17-ketosteroids.

Urinary estrogens. Results of biologic determination of estrogens in 24-hour collections of urine are indicated in Table 4. Excretion of estrogens

TABLE 4. URINARY EXCRETION OF ESTROGEN IN A CASE OF CHRONIC ADRENOCORTICAL DYSFUNCTION

Estrogen (rat units/24 hrs.)	Therapy	Comment
30	None	Average of 4 determinations
20	Corticosterone, 400 mg.	Determination on second day of therapy
0	Cortisone, 75 mg.	Determinations on fourth and fifth days of therapy
28	Cortisone, 80 mg.; ACTH, 120 mg.	Determinations on fourth and fifth days of therapy

was moderately increased even after a short period of administration of suppressive doses of corticosterone. However, when cortisone was given in sufficiently large doses and for sufficiently long periods to suppress the urinary 17-ketosteroids (Figs. 3 and 4), no estrogen was found in the urine. Subsequently, when cortisone and ACTH were administered concurrently, estrogens returned to pretreatment levels, as did the 17-ketosteroids and pregnanetriol.

COMMENT

The foregoing studies demonstrated that the abnormalities in electrolyte metabolism in this patient resulted from a deficiency of aldosterone. Our data do not support the possibility that the patient had primary renal disease. The mild polydipsia and polyuria appear to be adequately ex-

[3] The biologic activity of the preparation used was demonstrated in other patients.

plained by his great intake of salt and consequent large intake of water. Retention of urea occurred only when adrenocortical insufficiency was in evidence as the result of stress or a diminished intake of sodium, and it was corrected by adrenal replacement therapy. Plasma and urinary corticosteroid values were in the normal range, excretion of estrogens and 17-ketosteroids was increased slightly, and excretion of pregnanetriol was unequivocally high. The deficiency of aldosterone and the lack of response to exogenous ACTH suggest similarities to the cases of adrenocortical dysfunction already mentioned (1–13). The limited response to ACTH observed during concurrent administration of cortisone is indirect evidence that the steroid levels recorded under "resting" conditions were the result of a "maximal" effect of endogenous corticotropin on the adrenals. Unfortunately, direct observations on blood ACTH levels are not available.

The patient had experienced episodes typical of adrenal insufficiency twenty-one and fourteen years previously. Symptoms and physical findings had not changed appreciably in the interim. These considerations suggest that at the time of study the patient was not in a transitional phase on the way to the development of total adrenocortical insufficiency. In addition, we are unaware of any cases of proved Addison's disease in which the excretion of pregnanetriol was consistently increased. The patient reported on by Lichtwitz and associates (3) had high normal but not elevated values for 17-ketosteroids, and we too have seen some patients with Addison's disease who had low normal values for 17-ketosteroids.

These considerations suggest that our patient has adrenal hyperplasia similar to that reported in adult males (17–19). Unlike the situation in these cases, however, the increased excretion of steroids in our case was more moderate and was unresponsive to ACTH. There was no evidence of testicular enlargement or of any changes in the size of the testes during cortisone therapy, as was observed with Hedinger's (18) patient. Furthermore, the history of normal infancy, childhood and adolescence, the attainment of normal growth and development, and the demonstrated ability to produce sperm in the fifth decade of life make it doubtful that a significant degree of adrenal dysfunction had been present since birth. Hence, if he had "virilizing" adrenocortical hyperplasia, it would appear to be of the acquired type. On the other hand, it is possible that this patient was born with an enzymatic defect affecting the elaboration of adrenal steroids that may not have become quantitatively significant until adult life.

We can only conjecture about the mechanism by which the abnormalities in this patient were acquired. It is not known whether his adrenal cortices were in fact hyperplastic, as is suggested by similarities to the syndrome of virilizing adrenal hyperplasia with salt-losing features.

Neither is there anything more than conjectural evidence regarding possible enzymatic disturbances, among which a deficiency of 21-hydroxylase might be the major and most reasonably postulated one.

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INCREASED URINARY EXCRETION OF CATECHOL AMINES IN THE ABSENCE OF KNOWN CHROMAFFIN TUMORS

A SURVEY BY THE RABBIT AORTIC-STRIP METHOD OF 802 UNSELECTED, HOSPITALIZED PATIENTS*

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ABSTRACT

The urinary excretion of biologically active catechol amines was surveyed in 802 unselected, hospitalized patients. The rabbit aortic-strip method was used for assay. Normal values determined in 10 healthy, ambulatory medical students were 1.9 to 10.8 μg. (net) of norepinephrine equivalents per hour. Thirty-seven of the 802 patients excreted 12 μg. or more (net) of norepinephrine equivalents per hour in the urine and 2 of the 37 excreted as much as 72 μg. per hour. Increased excretion was more consistently observed in patients who had liver disease, infection of some type, a psychosis, or who died during the period of observation. Many of the patients had more than one illness, and various combinations of the foregoing factors were common in the patients with increased excretion. Possible explanations for these observations are briefly discussed. It is concluded that the isolated finding of "increased" excretion of catechol amines by the method herein employed probably should not be accepted as prima facie evidence of a pheochromocytoma. From a practical standpoint, this should not detract from using the test as a screening procedure for pheochromocytoma, provided the entire clinical situation is taken into account when interpreting the results.

PATIENTS with chomaffin tumors are known to excrete increased amounts of epinephrine and/or norepinephrine in the urine. During the past three years several patients have been observed who excreted catechol amines in amounts ordinarily considered to be in excess of normal. These patients did not consistently have hypertension, and in those who died the postmortem examination did not reveal a pheochromocytoma. No single disease could be indicted to account for these findings.

Using fundamentally the same method of bio-assay described in this study, Helmer (1) assayed the urine of 560 hypertensive patients for catechol amines. Two who excreted more than 12 μg. of catechol amines

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per hour were suspected of having a pheochromocytoma and were explored surgically, but no adrenal tumor was found. Normal values in Helmer's laboratory were considered to be 1.6 to 6.6 μg. per hour.

The present study was undertaken in order to elucidate some of the factors involved in excessive excretion of catechol amines in the absence of chromaffin tumors. The 24-hour urine specimens from 802 unselected, hospitalized patients were screened by means of a semiquantitative test for catechol amines. The urine of 37 of these patients yielded 12 μg. or more (net) of norepinephrine equivalents per hour. The observations on these 37 patients form the basis of this report.

METHOD

A modification of Furchgott and Bhadrakom's rabbit aortic-strip method (2) was used as described by Helmer (1). Urine was collected in 24-hour specimens and acidified to pH 3.5 with hydrochloric acid. Fifty-milliliter aliquots were frozen until two hours before the test, when they were allowed to thaw.

A spiral strip of aorta (4 mm. by 50 mm.) was prepared by sacrificing a rabbit by cervical fracture and quickly removing the aorta from the aortic ring to the iliac arteries. The strip was suspended vertically under 4 grams of tension in a Pyrex-glass chamber containing 40 ml. of Krebs-Henseleit (3) solution. The upper end of the muscle was connected by a pulley system to the pen of a direct-writing kymograph. The solution was maintained at 37° C. by means of a constant-temperature bath which circulated

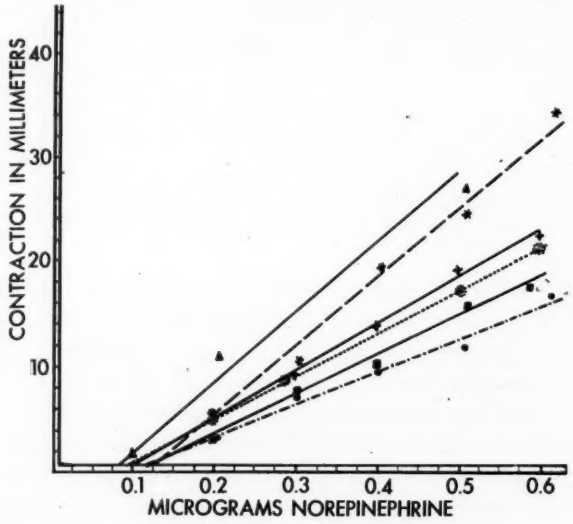

FIG. 1. The contraction of uniformly prepared strips of rabbit aorta induced by increasing doses of norepinephrine. The lowest limit of sensitivity is $0.1 \pm .025$ μg. of norepinephrine.

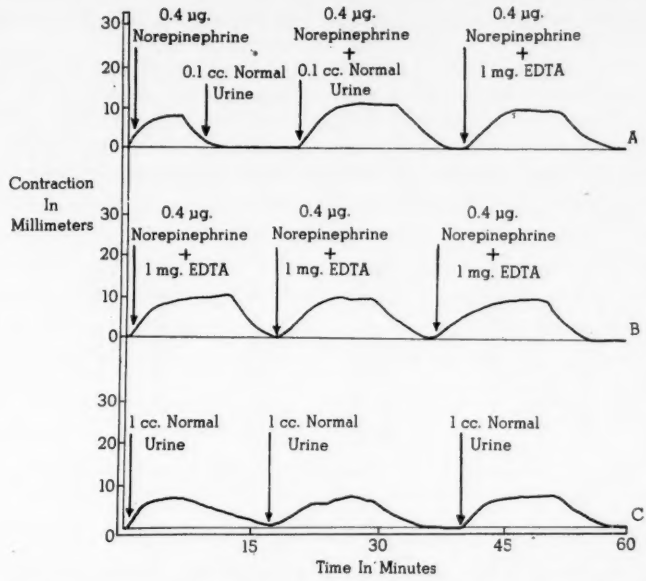

FIG. 2. **A**. The contraction of a strip of rabbit aorta induced by norepinephrine was potentiated by the addition of small amounts of normal urine and of EDTA. The potentiating action of the two substances was quantitatively similar but in this instance not identical. **B, C**. Reproducible responses to known quantities of norepinephrine and to identical samples of normal urine.

through an outer water-jacket of the chamber. A mixture of 95 per cent oxygen and 5 per cent carbon dioxide was continuously bubbled through the solution.

After the strip was allowed to incubate in the solution for three hours, a dose-response curve could be established according to the degree of contraction induced when known quantities of *l*-norepinephrine were added to the chamber (Fig. 1). One milligram of the disodium salt of ethylenediaminetetraacetic acid (EDTA) was added to the chamber with the standard, as suggested by Helmer (1). Muscle preparations were sufficiently sensitive for use if they inscribed a contraction of 11 mm. or more when 0.5 µg. of *l*-norepinephrine was added to the chamber. The smallest amount of free, biologically active *l*-norepinephrine which could be detected by these strips was consistently $0.1 \pm .025$ µg. (Fig. 1).

The average amount of urine excreted during thirty seconds of a total 24-hour period was added to the chamber when the pen was at the baseline. If a contraction was produced, the sample was considered to contain more than 0.1 µg. (net) of norepinephrine equivalents. If no contraction occurred or if the contraction was 2 mm. or less, the sample was considered to contain less than 0.1 µg. (net) of norepinephrine equivalents.

Quantitative determinations were made for all normal subjects and some of the patients. No special attempt was made to quantitate the catechol amine content of the urine from each of the 802 patients, except to show that the average volume of urine

excreted in thirty seconds contained more or less than 0.1 μg. (net) of norepinephrine equivalents (12 μg. (net) of norepinephrine equivalents per hour).

After each test the chamber was emptied, and fresh Krebs-Henseleit solution added from a heated reservoir. Contractions were considered to be produced by the catechol amines only if they were inhibited by 0.5 mg. of the adrenergic blocking agent, benzodioxane, added to the solution bathing the strip (Fig. 3). After completing a series of tests, 0.5 μg. of *l*-norepinephrine was again added to the chamber in order to demonstrate the viability of the muscle.

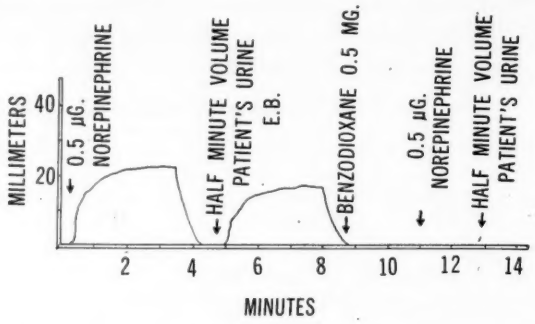

FIG. 3. Comparison of the contractions of a strip of rabbit aorta induced by 0.5 μg. of norepinephrine and by the urine of Patient E.B. who had lupus erythematosus and miliary tuberculosis. The patient excreted 49.2 μg. (net) of norepinephrine equivalents per hour. The effect was blocked by benzodioxane.

Reproducible responses were obtained when known quantities of norepinephrine or identical aliquots of urine were subjected to serial assays (Fig. 2, B and C). Very small amounts of urine were shown to potentiate the response to known quantities of norepinephrine to a degree that could not be accounted for by the catechol amine content of the urine itself (Fig. 2 A). The disodium salt of EDTA had a quantitatively similar potentiating effect (Fig. 1 A).

Chlorpromazine, hydralazine, and adrenolytic agents added *in vitro* blocked the response of the aortic strip to epinephrine and norepinephrine. Ganglionic blocking agents, rauwolfia derivatives, and thyroid substance did not interfere with the assay when added *in vitro*.

In general, the method used was almost identical to that described by Helmer (1). There were some differences, however. As opposed to Helmer's method, the physiologic solution bathing the strip contained no glucose, the capacity of the reaction chamber was 40 cc. rather than 20 cc., and the urine specimens were collected over a 24-hour period instead of a two-hour period.

RESULTS

Normal values for ambulatory subjects were determined in 10 healthy medical students. The mean urinary excretion was 6.05 μg. (net) of norepinephrine equivalents per hour, with a range of 1.9 to 10.8 μg.

The lowest limit of sensitivity of the aortic strips was consistently 0.1 μg. of norepinephrine. When the average amount of urine excreted in thirty

seconds was used for the test, any sample causing a contraction of the strip contained 0.1 μg. or more of pressor substance, and the hourly excretion was 12 μg. or more. This amount is slightly greater than the highest excretion observed in the normal subjects and was therefore selected as the dividing line between normal and "abnormal" excretion for purposes of screening a large population. The urine from patients with normal catechol excretion caused no contraction of the strip and further quantitation was not carried out. Eight hundred and two unselected, hospitalized patients were screened; 37 were detected who excreted 12 μg. or more (net) of norepinephrine equivalents per hour in the urine, and 2 of them excreted as much as 72 μg. per hour. Urinary catechol amines in these patients were estimated quantitatively (Table 1). In some instances the increased excretion of catechol amines seemed to bear no direct relationship to the nature of the illness (Table 2). In order to study the possible influence of each of several disorders upon urinary catechol amines the 802 patients were divided into 2 groups depending upon the level of excretion. The incidence of certain diseases in a group of 37 patients who excreted 12 μg. or more (net) of norepinephrine equivalents in the urine was compared to the incidence of the same diseases in a group of 765 patients who excreted less than this amount.

TABLE 1. THE TOTAL BIOLOGICALLY ACTIVE CATECHOL AMINE CONTENT OF URINE SPECIMENS FROM 37 PATIENTS WITH INCREASED EXCRETION OF PRESSOR SUBSTANCES BUT WITHOUT KNOWN CHROMAFFIN TUMORS. (WHEN SERIAL SAMPLES WERE COLLECTED FROM A GIVEN PATIENT ON SUCCESSIVE DAYS, THE MEAN VALUE WAS USED.)

Catechol amine excretion (μg./hr.)	No. of patients
12–20	2
20–30	7
30–40	11
40–50	7
50–60	2
60–70	5
70–80	3
Total	37

So far as we were able to determine, the 37 patients with the higher excretion were no different from the others with regard to age, sex, or race. However, a significant number of them suffered from infection, liver disease or a psychosis, or died during the period of observation. The data are summarized in Tables 3–5. It should be emphasized that many of these

TABLE 2. THE PERCENTAGE OF PATIENTS IN EACH DISEASE GROUP WHO EXCRETED μG. OR MORE OF EPINEPHRINE EQUIVALENTS PER HOUR IN THE URINE

Disease or disorder	No. of patients (total)	No. of patients excreting 12 μg. or more of norepinephrine equivalents per hr.	Percentage of patients* excreting 12 μg. or more of norepinephrine equivalents per hr.
Collagen	12	2	16.6
Psychosis	34	5	14.7
Death	114	16	14.0
Liver disease	63	8	12.7
Hematologic	36	4	11.1
Poorly controlled diabetes	37	4	10.8
Neoplasm	67	6	8.95
Infection	174	15	8.6
Heart disease; infarction	24	2	8.3
Cerebrovascular	69	5	7.2
Allergy	30	2	6.7
Diabetes, all types	170	11	6.5
Bowel; all organic types	68	4	5.9
Heart disease, all types	295	17	5.8
Renal	89	5	5.6
Other organic neuromuscular	57	3	5.3
Hypertension	221	11	5.0
Azotemia	164	5	3.0
Pregnancy	37	1	2.7
Pulmonary, all types	59	1	1.7
Peripheral vascular	62	1	1.6

* Many patients had more than one disease, which accounts for the overlap of percentages.

patients had more than one disease. This accounts for the overlap of percentages in Tables 2 and 3.

Some patients were receiving drugs which could possibly influence the rate of formation and/or inactivation of catechol amines, or which could interfere with the method of assay. These data are summarized in Table 6.

DISCUSSION

Excretion of catechol amines in normal subjects

The average excretion of biologically active catechol amines in the normal subjects herein reported was about three times that obtained by bioassay using the cat's blood pressure and chicken's rectal cecum as de-

TABLE 3. INCIDENCE OF CERTAIN DISEASES IN A GROUP OF PATIENTS EXCRETING 12 μG. OR MORE (NET) OF NORADRENALIN EQUIVALENTS PER HOUR IN THE URINE COMPARED TO THE INCIDENCE OF THESE DISEASES IN A GROUP OF PATIENTS EXCRETING LESS THAN THIS AMOUNT. (NORMAL EXCRETION: 1.9 TO 10.8 μG. PER HOUR)

Disease or disorder	Incidence in 37 patients* excreting, more than 12 μg. per hr. of norepinephrine equivalents (%)	Incidence in 765 patients* excreting less than 12 μg. per hr. of norepinephrine equivalents (%)	Significance of the difference between the 2 groups (*t* test)
Death	43.2	12.8	0.001
Infection	40.5	20.8	0.01
Liver disease	21.6	7.2	0.05
Psychosis	13.5	3.8	0.10
Neoplasm	16.2	7.97	>0.10
Hematologic	10.8	4.18	>0.10
Azotemia	13.5	20.8	>0.10
Poorly controlled diabetes	10.8	4.3	>0.10
Diabetes, all types	29.7	20.8	>0.10
Heart disease; all types	45.9	36.3	>0.10
Heart disease; infarction	5.4	2.9	>0.10
Cerebrovascular	13.5	8.4	>0.10
Other organic neuromuscular	8.1	7.0	>0.10
Collagen	5.4	1.3	>0.10
Allergy	5.4	3.7	>0.10
Bowel; all organic types	10.8	8.4	>0.10
Renal	13.5	10.9	>0.10
Hypertension	29.7	27.5	>0.10
Peripheral vascular	2.7	7.95	>0.10
Pregnancy	2.7	4.7	>0.10

* Many patients had more than one disease, which accounts for the overlap of percentages.

scribed by von Euler and Hellner (4), and twice that reported for the rabbit aortic-strip method by Helmer (1).

The method of von Euler and Hellner requires extraction of catechols with aluminum oxide. This was not done in our study; instead the whole urine was assayed. Recovery by extraction is about 70 per cent, and therefore results with this method tend to be lower than the actual values. On the other hand, normal urine contains substances which potentiate the action of epinephrine and norepinephrine (Fig. 1 A). When whole urine is used for assay, these substances tend to cause falsely high values. Helmer (1) found the potentiating action of urine to be similar to that of EDTA. To compensate at least in part for the potentiating action of

TABLE 4. PATIENTS WITH LIVER DISEASE WHO EXCRETED INCREASED AMOUNTS OF CATECHOL AMINES IN THE URINE

Patient		Diagnosis	Degree of liver disease	Comments
Name	Age (yrs.)			
R.S.	55	Laennec's cirrhosis	Severe	Liver enlarged 9 cm. Ruptured esophageal varices, 1938.
M.G.	75	Chronic passive congestion of liver	Severe	Liver enlarged 5 cm. Death 14 days after assay.
A.B.	71	Malnutrition; fatty liver	Severe	Liver enlarged 5 cm. Death 17 days after assay.
A.R.	32	Laennec's cirrhosis	Moderate	Liver enlarged 7 cm.
R.C.	53	Laennec's cirrhosis	Severe	Ascites and edema. Liver enlarged 9 cm. Patient died in hepatic coma 10 days after assay.
R.D.	59	Laennec's cirrhosis	Severe	Liver enlarged 7 cm.
A.M.	52	Hemochromatosis; cholangiolar carcinoma	Severe	Patient died in hepatic coma 19 days after assay.
G.S.	71	Chronic passive congestion of liver	Severe	Liver enlarged 8 cm. Postmortem: marked central necrosis.

urine, he suggested the addition of EDTA to the standard when determining the dose-response curve of aortic strips. This was done in our study. Nevertheless, our normal values were somewhat higher than Helmer's. Differences in the composition of the physiologic solution bathing the strip, the size of the reaction chamber, and the time interval during which the urine samples were collected may help to explain the discrepancy.

When whole urine is used for assay, there is no assurance that catechol amines are measured exclusively. Other unknown substances may be present which have some pressor activity. This is especially true when urine samples from patients having a variety of diseases are assayed. The values reported here represent total pressor activity, and the catechol

TABLE 5. INFECTIONS OBSERVED IN 15 OF 37 PATIENTS WITH INCREASED URINARY EXCRETION OF CATECHOL AMINES

Infection	No. of patients
Pneumonia	7
Chronic pyelonephritis	2
Cutaneous ulcers	2
Miliary tuberculosis	1
Staphylococcal septicemia	1
Pelvic inflammatory disease	1
Sinusitis	1
Total	15

amine content is expressed as *net* norepinephrine equivalents with this reservation in mind. In any event, amounts of biologically active pressor amines greatly in excess of our rather high normal values were found in a variety of illnesses.

TABLE 6. THE INCIDENCE OF ADMINISTRATION OF CERTAIN DRUGS IN A GROUP OF 37 PATIENTS WITH INCREASED AMOUNTS OF PRESSOR SUBSTANCES IN THE URINE COMPARED TO THE INCIDENCE OF ADMINISTRATION OF THE SAME DRUGS IN A GROUP OF 765 PATIENTS WITH NORMAL PRESSOR ACTIVITY

Drug	37 Patients who excreted 12 μg. or more (net) of norepinephrine equivalents per hr.		765 Patients who excreted less than 12 μg. (net) of norepinephrine equivalents per hr.	
	No. of patients who received the drug	Percentage of patients who received the drug	No. of patients who received the drug	Percentage of patients who received the drug
Insulin	7	18.9	94	12.3
Steroids or ACTH	4	10.8	21	2.7
Thorazine	4	10.8	35	4.6
Ephedrine	2	5.4	6	0.8
Hydralazine	1	2.7	39	5.1
Rauwolfia derivatives	1	2.7	62	8.1
Ganglionic blocking agents	0	0	6	0.8
Thyroid substance or triiodothyronine	0	0	4	0.5

Excretion of catechol amines in certain pathologic states

Agonal states. The increased excretion of norepinephrine-like substances in patients with infection, liver disease, or psychosis, and in those who died requires explanation. In terminal patients with increased excretion of catechol amines, the time of death was occasionally only a few hours, but more often five days to two weeks, after the urine sample had been collected. The act of dying may constitute a maximum stress, and the increased catechol excretion may be a nonspecific reflection of that stress. Although it is not possible to evaluate quantitatively all the physiologic mechanisms involved in death or survival, a disease severe enough to cause death may create a greater stress than a disease from which the patient partially or completely recovers.

An alternative explanation is that in the preterminal state of a disease there is deterioration of the enzyme systems which cause inactivation of pressor amines. Free, biologically active catechols may then be excreted in excess of normal in the urine.

Infections. It is of interest that of the 15 patients who had infections and increased excretion of catechol amines, 7 died, 4 were psychotic, and 1 had liver disease. In spite of the apparent correlation of infections and increased excretion of catechol amines, it is difficult to determine from our data to what extent an *isolated* infection influences catechol excretion, because in many instances the infections were complicated by other disorders.

Liver disease. Eight of the 37 patients with increased catechol amine excretion had liver disease (Table 6). A more specific mechanism may account for this observation. Guinea-pig liver slices are known to contain an amine oxidase which is capable of inactivating norepinephrine *in vitro* with the formation of catechol glycholic acid and catechol glycaldehyde (5). Human liver slices also inactivate both epinephrine and norepinephrine *in vitro* (6, 7). The inactivating properties are destroyed by inhibitors of amine oxidase (7). It has been shown that 95 to 98 per cent of infused norepinephrine is inactivated somewhere in the body (8), and it is possible that the liver plays a major role in this process. In the presence of hepatocellular damage, the inactivating mechanism (amine oxidase, peroxidase, conjugase, or some unknown mechanism) may be impaired and increased amounts of norepinephrine and epinephrine, free or conjugated, permitted to escape in the urine. It is difficult to say whether the liver disease itself or the act of dying is the major factor to account for the increased excretion. Nevertheless, this clinical finding correlates well with known experimental data and has not been previously reported.

Psychotic states. Five of the 37 patients with increased catechol excretion were psychotic. Although the association of psychotic states and in-

creased excretion of catechol amines has been known for some time, there is no proven explanation.

In 1952 Funkenstein and associates (9) suggested the probability of hypersecretion of epinephrine and norepinephrine in certain psychotic patients. Their conclusions were based on differences in the hypotensive response to methacholine in schizophrenic patients on the one hand, and manic depressive and involutional melancholic patients on the other. Elmadjian *et al.* (10) reported quantitative changes in the excretion of catechol amines in psychotic patients after the administration of lysergic acid. Leach, Angel, Heath and coworkers (11, 12) also investigated the metabolism of amines in schizophrenic patients. They believe there is a qualitative difference in the serum oxidase, ceruloplasmin, creating a faulty breakdown of catechol amines, and that there are associated quantitative changes in serum ceruloplasmin levels. Evidence to suggest that some psychoses are associated with disordered formation and/or inactivation of catechol amines is fragmentary, but results of the present study are consonant with the possibility.

Collagen diseases. Two of 12 patients with collagen diseases showed increased excretion of catechol amines. One of these patients died; the other was psychotic. Because of the small number of subjects, little significance can be attached to this observation.

Effect of certain drugs on the pressor activity of urine

Falsely low values for urinary catechol amines may have been found in some patients because chlorpromazine or hydralazine was being administered during the time that the specimens were collected. It is impossible to say on the basis of the present study to what extent the ingestion of these substances may decrease the values obtained by assay. Five patients who received these drugs still had increased pressor activity in the urine. The blocking effect, if present, was therefore not necessarily complete.

An increase in pressor activity could theoretically be caused by excessive production of epinephrine during periods of insulin-induced hypoglycemia. There was, however, no apparent tendency toward elevated excretion of catechol amines in diabetic patients in general or in diabetic patients receiving insulin.

The ingestion of ephedrine might also result in spurious high values. Although some patients receiving this drug had normal excretion, 2 had increased pressor activity that may have had no relation to the underlying disease.

It is clear that the interpretation of the test is obscured by the adminis-

tration of certain drugs. Therefore, when possible, all drugs should be discontinued at least twenty-four hours before the collection of urine is started.

In general, the isolated finding of "increased" excretion of catechol amines by the method herein employed probably should not be accepted as prima facie evidence of a pheochromocytoma. This is especially true when dealing with patients who have advanced liver disease, who are critically ill, psychotic, or have superimposed infections. However, the test is of practical value as a screening procedure for pheochromocytoma, provided the entire clinical situation is appraised when interpreting the results.

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DEMONSTRATION OF GROWTH HORMONE IN HUMAN PLASMA*

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ABSTRACT

A method is described for the concentration of growth hormone (GH) from human plasma. It is based on recovery experiments of human GH from human plasma and the demonstration of GH in plasma from patients with acromegaly. Human GH treated with trichloracetic acid is soluble in absolute ethanol and precipitates from the ethanol extract by pH-fractionation without any loss of biologic activity. A high concentration of GH was found in the plasma of patients with acromegaly but no activity could be detected in the plasma of normal subjects.

IN A previous report (1) the assumption was made that bovine and human growth hormone (GH) were chemically identical and consequently behaved similarly in human plasma. However, following the purification of GH from human pituitaries (2), Li and Papkoff (3) were able to show that the two GH preparations were chemically different. Furthermore, when each of the two hormones was added to human plasma and the mixture submitted to paper electrophoresis, the hormones migrated together with different plasma proteins (4). The human hormone migrated with the α_1 globulins whereas the bovine hormone migrated with the α_2 globulins. Consequently, a technique worked out for the concentration of bovine GH from human plasma might not be valid for the concentration of human GH from human plasma.

METHOD

Freshly drawn blood was cooled in ice-water and centrifuged. The plasma was removed and immediately treated with the same volume of 10 per cent trichloracetic acid (TCA). The precipitate formed was washed three times with 5 per cent TCA. After the last centrifugation the TCA precipitate was extracted three times with cold absolute ethanol; each time the volume of ethanol used was the same as the original plasma volume. The pH of the combined ethanol extracts was adjusted to 6.1 by careful addition of 0.1 *N* NaOH. The precipitate formed was suspended in cold water, dialyzed and lyophilized.

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TABLE 1. BIOASSAY BY THE TIBIAL TEST OF A HIGHLY PURIFIED PREPARATION OF HUMAN GROWTH HORMONE (71 E)

Total dose of human GH (71 E) (μg.)	No. of assay rats	Average width of tibial epiphysis (μ)
20	8	218 ± 2.6*
80	8	266 ± 7.1
Controls	12	174 ± 2.8

* Mean ± standard error of the mean.

The tibia epiphyseal test as proposed by Evans *et al.* (5) was used for the bioassay of the plasma fractions. The total dose of this fraction administered to each assay rat should not exceed 200 mg. A highly purified human pituitary GH preparation (71 E) was used as a reference standard (Table 1 and Fig. 1). The average width of the tibial epiphysis of the control rats was 174 micra; a width less than 180 micra was considered a negative finding.

RESULTS

Recovery of added human GH from human plasma

Human GH was dissolved in a small amount of physiologic saline and added slowly (at 4° C. with stirring) to lyophilized human plasma which had been dissolved in physiologic saline to the original amount of the sample. The plasma with the added GH was immediately fractionated ac-

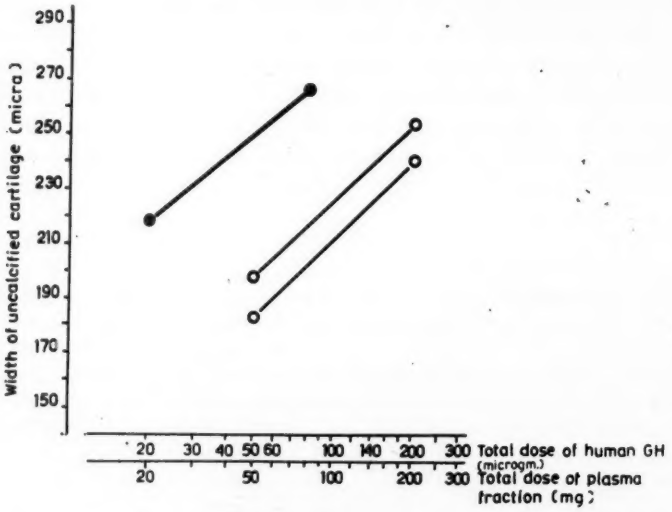

FIG. 1. Bioassay by the tibia test of fractionated plasma of a patient with acromegaly (A.E.). The thick line represents the standard GH preparation (71E).

cording to the method described. Aliquots of the active plasma fraction were injected into the assay rats. As seen in Table 2, the recovery varied between 32 and 110 per cent. The low recovery might be explained by the fact that in Experiments 708 and 287 the final pH deviated from that used in the described method. Thus, in Experiment 708 the pH of the ethanol extract was brought to 5.4 instead of to 6.1, and in Experiment 287 the pH was brought beyond 6.1. In the remaining experiments in which the pH fractionation was carried out close to pH 6.1 the recovery was almost 100 per cent.

TABLE 2. RECOVERY OF ADDED HUMAN GROWTH HORMONE (71 E) FROM FRACTIONATED HUMAN PLASMA

Expt. No.	Amount of lyophilized plasma (mg.)	Amount of added human GH (mg.)	pH	Amt. of protein in active fraction (mg.)	Total dose of fractionated plasma per assay rat (mg.)	No. of assay rats	Avge. width of tibial epiphysis (μ)	Recovery of added human GH (%)
708	1450	1.0	5.4	498	25	5	207 ± 3.2	32
284	1300	0.1	6.1	685	171	4	223 ± 6.2	96
286	1300	0.4	6.2	696	174	4	277 ± 7.2	110
285	1300	0.1	6.2	688	172	4	213 ± 4.2	76
287	1300	0.4	6.4	634	159	4	244 ± 6.0	45

It is possible that the relatively high recovery of added GH in some of the experiments may have been due to substances in plasma such as thyrotropic hormone which acted synergistically with GH, or to GH in the plasma which alone did not yield a positive result with the tibia test.

Various amounts of the plasma fraction were administered to the assay rats, with or without 20 micrograms (μg.) of the human GH preparation (Table 3). The data in the table show that the plasma fraction did not increase the effect of GH on the width of the tibial epiphysis and did not alone elicit a positive response to the test.

Concentration of GH in the plasma of patients with acromegaly

The plasma of 5 patients was fractionated (Table 4). The high concentration of GH in the plasma of Patient A.E. made it possible to perform an assay with more than one dose level. Plasma from this patient was tested by a four-point assay with 2 dose levels of the plasma fraction and 2 dose levels of the Standard GH preparation (71 E). As seen in Figure 1 the curves obtained were almost parallel. Following the treatment of this

TABLE 3. THE EFFECT OF VARIOUS AMOUNTS OF FRACTIONATED HUMAN PLASMA ON THE TIBIAL TEST

Total dose of human GH (71 E) (μg.)	Total dose of fractionated human plasma (mg.)	No. of assay rats	Avge. width of tibial epiphysis (μ)
20	0	8	221 ± 5.3*
20	50	8	229 ± 4.9
20	200	8	225 ± 4.1
0	50	8	171 ± 2.9
0	200	8	169 ± 3.4

* Mean ± standard error of the mean.

patient (x-ray therapy to the pituitary), no GH activity could be detected in the plasma. The second patient (O.H). suffered from mild acromegaly which had been fairly stationary for a couple of years, and the concentration of GH in the plasma was considerably lower than in the first patient. Patients L.B. and Y.C. had relatively mild forms of the disease, and in them the plasma level of GH was around 400 μg. per 100 ml. Patient A.H.

TABLE 4. PLASMA LEVELS OF GROWTH HORMONE IN PATIENTS WITH ACROMEGALY

Plasma from Patient A.E. was drawn and tested on 3 different occasions, twice before and once after pituitary X-ray therapy. Plasma from Patient A.H. was drawn and tested on 2 different occasions after hypophysectomy.

Patient		Diagnosis	Treatment	Amt. of plasma (ml.)	Amt. of protein in active fraction (mg.)	Total dose of fractionated plasma per assay rat (mg.)	No. of assay rats	Avge. width of tibial epiphysis (μ)	Amt. of GH per 100 ml. of plasma (μg.)
Initials	Age (yrs.) & Sex								
A.E.	28 F	Acromegaly	0	40	1416	50	4	183 ± 3.4	570
						200	4	238 ± 7.2	655
			0	32	1310	50	4	198 ± 3.6	1230
						200	4	254 ± 8.1	1160
			X-ray therapy to pituitary	16	388	97	4	176 ± 5.2	—
O.H.	35 M	Acromegaly	0	25	728	182	4	212 ± 2.1	288
L.B.	68 F	Acromegaly	0	22	720	180	4	215 ± 6.1	364
Y.C.	48 M	Acromegaly	0	13	300	60	5	194 ± 2.6	440
A.H.	30 F	Acromegaly	Hypophysectomy	22	640	160	4	176 ± 1.9	—
			Hypophysectomy	21	510	127	4	168 ± 2.4	—

had been operated upon three months earlier and a tumor of the pituitary had been removed. The average width of the tibial epiphysis in the assay rats was less than 180 micra, and no significant elevation of the GH level in the plasma of this patient could be found.

Concentration of GH in the plasma of normal subjects

Plasma from a large number of normal persons was examined. In general, the total dose of the plasma fraction per assay rat did not exceed 200 mg., which roughly corresponded to an aliquot of 12 to 14 ml. of whole blood. In no case did the average width of the tibial epiphysis of the assay rats exceed 180 micra. It might be possible to administer larger amounts of the plasma fraction to each assay rat and eventually obtain measurable levels. However, doses that were too large injured the rats and consequently spoiled the results of the assay.

DISCUSSION

Elevated levels of a growth-promoting factor in untreated plasma of a patient with acromegaly have been reported by Kinsell *et al.* (6), and in fractionated plasma by Gemzell *et al.* (1). Due to the low concentration of this factor in plasma, the bioassay was performed at one dose level only, and thus the identification of the factor as GH was uncertain. By the technique used in this study, it was possible to concentrate the growth-promoting factor in the plasma of a patient with acromegaly to such an extent that a bioassay with two dose levels could be performed. As the curve obtained for the plasma fraction and the curve obtained for the Standard GH preparation were almost parallel, the identification of the growth-promoting substance as GH seemed more significant.

By the technique used in this study, the GH was found to be in a plasma fraction that consisted of about 50 per cent of the total plasam protein. When the procedure yielded a plasma fraction that was less than 50 per cent of the proteins, the recovery of added GH was significantly lower. Thus, the fractionation technique was not suitable for purification of the GH from plasma to any further extent.

Korner and Debro (7) have shown that the ethanol extract of TCA-treated plasma contains mainly albumin. When the plasma fraction of this study was applied to zone electrophoresis on starch it migrated as a single protein with the same rate as albumin. A large number of experiments were performed in order to separate the GH from the albumin in the plasma fraction. It was possible to separate the two substances by chromatography on Amberlite IRC-50 (XE-97) columns or by zone electrophoresis on starch in a Veronal buffer at pH 8.3, but only when a relatively large amount of GH was added to plasma. When normal plasma or plasma from

patients with acromegaly was treated by these methods, however, a separation was not obtained.

Acknowledgment

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RESISTANCE TO INSULIN DUE TO NEUTRALIZING ANTIBODIES*

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ABSTRACT

A case of insulin-resistant diabetes mellitus due to neutralizing antibodies is reported in detail; studies on sera from 6 other insulin-resistant cases are also reported. In the case which was studied in detail, the following observations were made: (a) The patient exhibited resistance to ox, pig and sheep insulins. A total of 2100 units of ox insulin, 1100 of which were administered intravenously, was given in the course of one day without any effect on the high blood sugar level. (b) Allergy to insulin was demonstrated by urticaria, eosinophilia and anaphylactic reaction following intravenous administration of insulin. (c) Insulin-neutralizing antibodies were present in the serum. They protected starving mice against convulsions ordinarily induced by ox, pig, sheep, horse, monkey, whale, fish (ling), chicken and human insulin; they did not protect against convulsions induced by guinea-pig insulin. (d) Insulin was recoverable in active form from the plasma after an intravenous injection; anaphylaxis, but not lowering of the blood sugar level, followed the injection. (e) Insulin mixed with the serum of this patient was precipitated in the cold by alcohol (25 per cent), whereas insulin mixed with normal serum was not so precipitated. (f) The diabetes was controlled by a low caloric intake with a relatively high proportion of fat in the diet.

INTRODUCTION

SEVERAL investigators have reported that serum from certain insulin-resistant human subjects is capable of neutralizing the hypoglycemic activity of insulin (1–10). Lowell and associates (11, 12) presented significant evidence in support of the assumption that such neutralization is consistent with antigen-antibody reaction. They showed, for example, that rabbits could be rendered resistant to insulin by the injection of either ox or pig insulin in Freund's adjuvant, and that the induced resistance was directed toward crystalline ox or pig insulin but not toward the endogenous insulin of the resistant rabbit, nor toward a preparation of human insulin.

Moloney and co-workers (13, 14) using experimental animals likewise showed that insulin manifests the characteristics of a true antigen, although a weak one, as compared with such antigens as diphtheria and

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tetanus toxoids. By repeated injections of crystalline pig or ox insulin with adjuvant, they were able to induce resistance to insulin in the mouse, guinea pig, rabbit, sheep and horse. The insulin-resistant animals showed no signs of diabetes, and hence it was concluded in agreement with Lowell *et al.* that the anti-insulin produced in the experimental animal did not neutralize that animal's endogenous insulin. However, anti-insulin in the serum of the guinea pig, rabbit, sheep or horse neutralized (as shown by the mouse test) insulin extracted from the pancreas of all species tested (pig, ox, rabbit, sheep, horse, monkey, whale, chicken and man) except that of the guinea pig. Thus it would appear that the process of extraction had altered the immunologic specificity of all the insulins extracted with the possible exception of guinea-pig insulin. It was found that insulin may be freed from a neutral insulin + anti-insulin serum complex by acidification to pH 2.3 (13). This observation serves as one criterion of antigen-antibody combination and distinguishes neutralization of insulin referred to here from destruction of insulin by enzyme action.

Although the anti-insulin that was induced in the guinea pig did not neutralize guinea-pig endogenous insulin, it was capable of neutralizing endogenous mouse insulin, as shown by the fact that repeated injections of guinea-pig serum of high anti-insulin titer induced fatal diabetes in the mouse (13).

Recently a patient with acquired resistance to commercial insulin, due to neutralizing antibodies, was investigated at the Toronto General Hospital. Clinical resistance was shown to ox, pig and sheep insulins. By the mouse convulsion test, the pattern of neutralization of the serum of this patient was identical with that observed with the serum of certain animals rendered resistant to insulin.

In addition, during the course of this study, we received serum specimens from 6 other patients who manifested resistance to insulin. By the mouse convulsion test these sera neutralized insulin. Results in these 6 cases are reported after a more detailed presentation of the results obtained in the first case.

CASE REPORT[1]

Case 1

Mrs. E. T., a Negress aged 62, was admitted to the Toronto General Hospital on November 26, 1956. Seven years previously, glucosuria had been discovered when she consulted a physician because of shingles. She refused treatment and sought no further medical advice until eighteen months before admission, when she attended the Out-Patient Department with acute otitis media. For the previous six months she had been

[1] We are indebted to Dr. Jonathan C. Sinclair, Physician, Toronto General Hospital, for permission to study and report this case.

losing weight, and had polyuria and polydipsia. She weighed 109 pounds. No retinopathy was noted; blood pressure was 125/75 mm. Hg; there were no arterial pulses in the right foot. Vibration sense was impaired in both legs, and ankle jerks were absent.

Urinalysis showed the presence of sugar +++, but no acetone. The level of fasting blood sugar was 372 mg. per 100 ml.

She was treated as an out-patient in the Diabetic Clinic, with a diet of protein 60 Gm., fat 110 Gm. and carbohydrate 125 Gm., and 20 units of protamine zinc insulin (PZI) daily. The diabetes was fairly well controlled for the next ten months. Then she stopped coming to the Clinic, but continued to take insulin sporadically until six months before admission to hospital, since which time she had neither taken insulin nor observed dietary restrictions. Two months before admission, 2 small areas of gangrene appeared, one at the side of the right great toe where she had pared a callus, and the other over the right middle toe which had been pressing against the end of an ill-fitting shoe. She came to hospital because of pain in these toes.

On admission, her weight was 108 pounds and her temperature was normal. She looked well and, when off her feet, was in no distress. Many Ballantyne aneurysms with small flame-shaped hemorrhages and perimacular exudates could be seen in the optic fundi. Blood pressure was 160/90. No arterial pulse below the popliteals could be felt. There were 2 frankly gangrenous areas in her right foot.

The urine had a specific gravity of 1.034 and contained sugar +++, but no ketones or albumins; microscopic examination showed 3 to 4 leukocytes per high-power field, and an occasional granular cast. The postprandial blood sugar level on the afternoon of admission was 380 mg. per 100 ml. On November 26, 1956, the hemoglobin level was 12.5 Gm. per 100 ml. and the leukocyte count was 12,000 per cu. mm., with 66 per cent neutrophils, 22 per cent lymphocytes, 3 per cent monocytes and 9 per cent eosinophils. A dose of insulin was given before the differential count was made. On December 8, 1956 and January 7, 1957, the percentages of eosinophils were still 9 and 14, respectively. Allergy to insulin probably accounted for the moderate eosinophilia. Paper strip electrophoresis of serum drawn on December 30, 1956, showed a gamma globulin level of 1.45 Gm. per 100 ml., which is toward the upper limit of the normal range.

Development of insulin resistance. Treatment and laboratory findings are summarized in Table 1.

The diet that was prescribed contained protein 75 Gm., fat 100 Gm. and carbohydrate 125 Gm. The patient received 15 units of Connaught crystalline zinc ox insulin the first evening in hospital. The fasting blood sugar level on the next morning was 186 mg. per 100 ml. Crystalline insulin was continued in doses of 10 to 20 units once or twice a day for the next three days. On November 30, 1956, a dose of 20 units of PZI was given at 8 A.M.; the fasting blood sugar level was 150 mg. per 100 ml. Because of continuing glucosuria throughout the day, a dose of 20 units of crystalline insulin was added at 10 P.M. At 1 A.M. the patient complained of feeling sweaty, cold and nervous. These symptoms were relieved by orange juice. A blood sugar estimation was not obtained until the next morning, when it was found to be 106 mg. per 100 ml. Again, a dose of 20 units of PZI was given at 8 A.M. Glucosuria was noted during that day, but there was no sugar in the urine passed at 8 o'clock the next morning.

From then on, nearly constant glucosuria was observed in spite of increasing doses of insulin. The dose of PZI was raised to 25 units. Fasting blood sugar levels on the next four days were 279, 210, 242 and 296 mg. respectively per 100 ml. On December 6, a total of 90 units of crystalline insulin was given during the day. Fasting blood sugar concentration on the next morning was 203 mg. per 100 ml. The dosage of insulin was

TABLE 1. DOSAGE OF INSULIN AND BLOOD SUGAR LEVELS
(CASE 1)

Date	Insulin		Blood sugar level* (mg./100 ml.)	Comments
	Type of preparation	Total daily dose (units)		
1956				
Nov. 26	Solution (ox crystals)	15	—	Diet: P 75; F 100; C 125.
Nov. 27	Solution (ox crystals)	25	186	
Nov. 28	Solution (ox crystals)	20	120	
Nov. 29	Solution (ox crystals)	30	154	
Nov. 30	Solution (ox crystals)	20	150	
	Protamine zinc (ox and pig)	20	—	
Dec. 1	Protamine zinc (ox and pig)	20	106	Hypoglycemic reaction at 1:30 A.M.
Dec. 2	Protamine zinc (ox and pig)	20	—	
Dec. 3	Protamine zinc (ox and pig)	25	279	
Dec. 4	Protamine zinc (ox and pig)	25	210	
Dec. 5	Protamine zinc (ox and pig)	25	242	
Dec. 6	Solution (ox crystals)	90	296	
Dec. 7	Solution (ox crystals)	370	203	
Dec. 8	Solution (ox crytsals)	195	236	
Dec. 9	Solution (ox crystals)	125	—	
Dec. 10	Solution (ox crystals)	70	190	
Dec. 11	Solution (ox crystals)	70	187	
Dec. 12	Solution (ox crystals)	195	218	
Dec. 13	Solution (ox crystals)	225	250	
Dec. 14	Solution (ox crystals)	300	236	
Dec. 15	Solution (ox crystals)	330	243	
Dec. 16	Solution (ox crystals)	270	—	
Dec. 17	Solution (ox crystals)	315	202	
Dec. 18	Solution (ox crystals)	380	190	
Dec. 19	Solution (ox crystals)	350	—	
Dec. 20	Solution (ox crystals)	300	204	
Dec. 21	Solution (ox crystals)	270	—	
Dec. 22	Solution (ox crystals)	190	—	
Dec. 23	Solution (ox crystals)	200	—	
Dec. 24	Solution (ox crystals)	400	200	B.S.* det'd at 11:30 A.M.
Dec. 25	Solution (ox crystals)	400	312	B.S. det'd at 11:45 A.M.
Dec. 26	Solution (ox crystals)	850	—	
Dec. 27	Solution (ox crystals)	1,000	250	B.S. det'd at 2 P.M.
Dec. 28	Solution (ox crystals)	2,000	259	

* Blood sugar (B.S.) levels were determined by the method of Folin and Wu (15) on specimens taken at 8 A.M. (fasting), unless otherwise stated. (Normal range, fasting, 80–120 mg./100 ml.)

TABLE 1. *Continued*

Date	Insulin		Blood sugar sugar* (mg./100 ml.)	Comments
	Type of preparation	Total daily dose (units)		
Dec. 29	Sheep	400	208	
Dec. 30	Sheep	1,000	250	
Dec. 31	Solution (ox crystals) 1,000 μ., intramusc. 1,100 μ., intraven.	2,100	190	
1957				
Jan. 1	—	—	266	
Jan. 2	—	—	250	Tolbutamide (3 Gm. daily for 5 days, Jan. 2–6)
Jan. 3	—	—	244	
Jan. 4	—	—	—	
Jan. 5	—	—	198	
Jan. 6	—	—	—	
Jan. 7	—	—	258	Tolbutamide (1.5 Gm. daily for 10 days, Jan. 7–16)
Jan. 8	—	—	—	
Jan. 9	Isocyanate†	60	218	
Jan. 10	Isocyanate†	100	208	
Jan. 11	Solution (ox crystals)	175	230	Prednisone, 40 mg.
Jan. 12	Solution (ox crystals)	150	362	Prednisone, 10 mg.
Jan. 13	—	—	256	
Jan. 14	—	—	251	Diet changed to: P 60; F 70; C 75.
Jan. 15	—	—	210	
Jan. 16	—	—	200	
Jan. 17	—	—	200	
Jan. 18	—	—	208	
Jan. 19	—	—	170	
Jan. 20	—	—	162	
Jan. 21	—	—	153	
Jan. 22	—	—	169	
Jan. 23	—	—	193	
Jan. 24	—	—	167	
Jan. 25	—	—	159	
Jan. 26	—	—	203	Diet changed to: P 30; F 100; C 50.
Jan. 27	—	—	173	
Jan. 28	—	—	139	
Jan. 29	—	—	118	
Jan. 30	—	—	109	

† A conjugate of phenyl isocyanate and insulin prepared by Novo Laboratory, Denmark.

raised to 300 units daily (crystalline) in fractional doses, yet the fasting blood sugar level remained around 200 mg. per 100 ml., and there were ketone bodies in the urine.

On the morning of December 17, 1956, an insulin tolerance test was performed. A dose of 75 units of crystalline insulin was given hypodermically at 8 A.M., and breakfast was omitted. The fasting blood sugar concentration increased from 202 mg. to 227 mg. per 100 ml. four hours later.

The dosage of crystalline insulin was increased to a total of 2000 units per day, 500 units being given hypodermically every six hours. Following these injections transient urticaria was noted, which was usually maximal at the sites of injection. However, wheals also appeared on the trunk where no insulin had been given.

Connaught protamine zinc insulin contains mixed ox and pig insulins. Connaught crystalline insulin is derived from ox insulin. Dr. Romans of the Connaught Laboratories made available some sheep insulin, which was given for two days. No effect was obtained even when a dose of 750 units was given on the second day.

The suggestion has been made that in some cases of insulin resistance, there may be local inactivation at the site of injection (16). In such a case, intravenous administration of insulin might be effective. Accordingly, a test dose of 100 units of crystalline ox insulin was given intravenously on December 31, 1956, at 12.30 P.M. It produced transient urticaria, but it did not lower the blood sugar level, as shown in a sample taken at 2.30 P.M. At 3.00 P.M., an injection of 1000 units of crystalline insulin was given intravenously. This immediately produced a mild anaphylactic reaction. As the needle was being withdrawn it was noted that the patient had become pale, was perspiring profusely, and appeared acutely ill. Her systolic blood pressure fell to 80 mm. Hg. She complained of pain in the back of her neck and at the site of injection. Soluble hydrocortisone (50 mg.) was given intravenously, followed immediately by 0.5 ml. of epinephrine hypodermically. Thereafter she continued to improve throughout the day. Half an hour after the injection, her blood pressure was 100 systolic. There was no bronchospasm or cyanosis at any time. The next morning the concentration of blood sugar was 266 mg. per 100 ml.

On January 2, 1957, a course of tolbutamide was started in a dosage of 3 Gm. daily for five days; then the dose was decreased to 1.5 Gm. daily for the next ten days. There was no effect on the blood sugar level or on glucosuria.

A Danish preparation of isocyanate insulin[2] was tried at the same time as tolbutamide was being given. It was hoped that this chemically altered insulin might be sufficiently changed in specificity not to be neutralized. However, it was not effective.

Corticotropin and cortisone have been given to diabetics when resistance has been thought to be due to specific antibodies. Both favorable (17) and unfavorable (5) results have been reported following this type of treatment.

Because of the possibility of beneficial effect, our patient was given 40 mg. of prednisone together with 175 units of crystalline insulin on January 11, 1957. The next morning the concentration of fasting blood sugar had risen to a peak of 362 mg. per 100 ml., and the carbon dioxide combining power of the plasma had fallen to 36 volumes per cent. Because the diabetes had become worse, prednisone was discontinued that day after the first 10-mg. dose had been given. The next morning, the fasting blood sugar level was 250 mg. per 100 ml., and the carbon dioxide combining power was 52 volumes per cent.

It was decided to attempt to control the hyperglycemia by restricting the daily caloric intake to about 1200 calories. With the new diet containing protein 60 Gm., fat 70 Gm. and carbohydrate 75 Gm., fasting blood sugar levels were lower, but still above the

[2] See second footnote in Table 1.

normal range. Finally with a new isocaloric diet, low in carbohydrate and high in fat (protein 30 Gm., fat 100 Gm. and carbohydrate 50 Gm.), normal concentrations of fasting blood sugar were achieved. Since the diabetes could be controlled by diet alone, it can be concluded that the patient was producing insulin, and that this endogenous insulin was not being neutralized by her anti-insulin.

Because of family problems, on January 30, 1957, the patient left the hospital against advice. The gangrenous areas in the toes had remained about the same size as they were on admission. She did not adhere to her diet while at home. One month later she was readmitted because of progression of the gangrene, which had led to cellulitis in neighboring tissues. An amputation below the knee was performed, and the wound healed well. Although no insulin was given, the diabetes remained well controlled with a diet of protein 30 Gm., fat 100 Gm. and carbohydrate 50 Gm., before and after the operation.

IMMUNOLOGIC FINDINGS

Neutralization of insulin by immune serum

When the increasing need for insulin was noted in this patient, the neutralizing activity of her serum was tested by the mouse-convulsion assay procedure (13). Tests were carried out by treating starved mice with injections of either undiluted or diluted serum mixed with an amount of insulin which, when injected with physiologic saline or normal serum caused convulsions in 95–100 per cent of the mice. The results of a typical test are shown in Table 2.

Anti-insulin titers of several specimens of the patient's serum are recorded in Table 3.

TABLE 2. INSULIN NEUTRALIZATION TEST (CASE 1)

Each mouse received an injection containing 0.5 ml. of a mixture of equal volumes of a solution of crystalline ox insulin (0.2 μ./ml.) and serum dilution as shown in the Table.

Mixture injected per mouse		No. of mice	No. of mice with convulsions	Comment
Units of insulin	Vol. & dilution of serum			
0.05 +	0.25 ml. undil.	6	0	Insulin neutralized
0.05 +	0.25 ml., $\frac{1}{2}$ dil.	6	6	Insulin not neutralized
0.05 +	0.25 ml., $\frac{1}{4}$ dil.	6	6	Insulin not neutralized

These results indicate (by the mouse-convulsion criterion):

1) That 0.25 ml. of undiluted serum neutralizes 0.05 unit of insulin, or 1.0 ml. of undiluted serum neutralizes 0.2 unit of insulin.

2) That 0.25 ml. of $\frac{1}{2}$ dilution of serum (which is equivalent to 0.125 ml. of undiluted serum) does not neutralize 0.05 unit of insulin, or 1.0 ml. of undiluted serum does not neutralize 0.4 unit of insulin. Hence the neutralizing titer of the serum specimen is $>0.2<0.4$ unit insulin per ml.

TABLE 3. ANTI-INSULIN TITER OF PATIENT'S SERUM (CASE 1)

Date	Insulin injected	Units of insulin neutralized per ml. of serum
1956		
Dec. 19	Increasing doses of insulin (ox, pig, sheep) subcut. in the period Nov. 26 to Dec. 30, 1956	>0.2 <0.4
Dec. 30		>0.4 <0.8
Dec. 31	Ox insulin, 1,000 μ, intramusc. Ox insulin, 1,100 μ. intraven.	
1957		
Jan. 8	—	>1.6 <3.2
Jan. 9	Isocyanate insulin, 60 μ. subcut.	
Jan. 10	Isocyanate insulin, 100 μ. subcut.	
Jan. 11	Ox insulin, 175 μ. subcut.	>0.8 <1.6
Jan. 12	Ox insulin, 150 μ. subcut.	
Jan. 25	—	>0.8 <1.6
Feb. 25	—	>0.2 <0.4
Apr. 3	—	>0.2 <0.4
Sept. 25	—	<0.2

Dixon and associates (18) have reported that the half-life of gamma globulin in humans is in the order of 20 days. If one assumes that this is the order of the half-life of antibodies, then from the results shown in Table 3 one can conclude that in the period Feb. 25 to April 3, 1957, the patient was still producing antibodies to insulin, since even in 35 days (Feb. 25 to April 3) antibody level had not decreased by half.

Specimens of serum from the patient neutralized not only crystalline ox and pig insulin, but also extracted insulin from rabbit, sheep, horse, monkey, whale, fish (ling), chicken and human pancreas. However, extracted guinea-pig insulin was not neutralized.

The nature of the inactivation of insulin by antibody

Moloney and Coval (13) showed that insulin could be released from a neutral insulin+anti-insulin (guinea-pig serum) mixture by acidification to pH 2.5, thus indicating that neutralization was not due to destruction of insulin.

It was also true for Case 1 in our study that neutralization of insulin by the patient's serum was not due to destruction. This was demonstrated by Dr. G. A. Wrenshall, who extracted insulin from the blood plasma of this patient following the intravenous injection of 1100 units of crystalline insulin. It will be recalled that the injected insulin did not have any hypoglycemic effect, but that it did produce an anaphylactic reaction. The

plasma specimens were deep-frozen. Later they were extracted with acid alcohol (19) and assayed for insulin by the mouse-convulsion method. We are indebted to Dr. Wrenshall for permission to report his findings.

Results are summarized in Table 4.

The foregoing findings are consistent with the conclusion of Berson and Yalow (20) that insulin and insulin-binding antibody form a complex which is in reversible equilibrium with the uncomplexed species.

The rate of disappearance of injected insulin from the blood plasma of our patient, a drop from 324 to 126 units in fifteen hours, probably bears

TABLE 4. INSULIN EXTRACTED FROM PLASMA AFTER INTRAVENOUS INJECTION OF 1,100 UNITS

Hours after injection of insulin	Insulin (μ./ml. plasma)	Total units
1	0.18	$1{,}800 \times 0.18 = 324$
16	0.07	$1{,}800 \times 0.07 = 126$

Calculated plasma volume = 1,800 ml. Body weight = 45 Kg. Assumed value for plasma concentration = 41 ml./Kg. body weight.

little relation to the rate at which insulin is normally metabolized, but depends rather on factors having to do with removal from plasma, and disposal of antigen bound to antibody.

It is pertinent here to cite the work of Welch *et al.* (21) and Berson *et al.* (22), who found that I^{131}-labeled insulin disappeared more slowly from the plasma of insulin-treated subjects than from the plasma of non-insulin-treated subjects.

Anti-insulin titers of the 6 additional cases referred to in the introduction are reported in Table 5. It should be noted that, in spite of the daily administration of insulin, the anti-insulin titer in Cases 3, 4 and 7 decreased to undetectable levels. In Case 7 serum-neutralizing activity was present before acidosis developed. With the onset of acidosis there was a marked increase in the requirement of insulin, which could be explained by the sudden appearance of an insulin antagonist other than antibody, such as has been reported by Field and Stetten (23) as occurring in diabetic acidosis. The sudden drop in anti-insulin titer could be explained by neutralization due to a very large dose of insulin (5000 units) given over a few hours.

Among the cases listed in Table 5, hyperglycemia persisted during treatment with the recorded dosages of insulin, except in Case 4; thus, a correlation cannot be expected between dosage of insulin and anti-insulin level. This does not imply that anti-insulin resistance cannot be at least

TABLE 5. ANTI-INSULIN TITERS IN 6 INSULIN-RESISTANT DIABETICS*

Case No.	Patient	Date	Units of insulin neutralized per ml. of serum	Maximum total† daily dose of insulin (units)	Comment			
					Allergy	Ketosis	Clinical improvement with adrenal steroid therapy	Duration of resistance to insulin
		1957						
2	M.V.	August	0.8	260	No	No	No	Temporary
3	J.B.	Nov. 3	0.4	600	Urticaria	No	No	Temporary
		Dec. 19	<0.2					
4	R.D.	Dec. 10	>0.4<0.8	200	No	No	None given	Fluctuating
		1958						
		Jan. 17	0.4					
		Mar. 11	>0.2<0.4	‡				
		Mar. 24	0.2					
		Apr. 2	>0.2<0.4					
		Jun. 21	<0.2					
5	K.R.	Mar. 20	0.2	480	No	Yes	Yes	Fluctuating
		May 22	0.4					
		Jun. 5	0.4					
6	C.F.	Jun. 5	0.2	170	No	No	None given	Persistent
		Aug. 21	0.2					
7	M.H.	Aug. 7	0.4	120	No	Yes	No	Fluctuating; patient died Aug. 16, 1958, of cerebral infarction
		Aug. 11	>0.2<0.4					
		Aug. 13	<0.2	5000				
		Aug. 16	<0.2	100				

* We are indebted to the following physicians for serum specimens and for permission to publish the data: Dr Robert C. Dickson, Halifax—Case 2; Dr. Allan Gold, Montreal—Case 3; Drs. Ian Macdonald and Bernard S. Leibel, Toronto—Case 4; Dr. Joan Vale, Toronto—Case 5; Dr. Eva M. Macdonald, Toronto—Case 6; and Dr. D. A. Stinson, Toronto—Case 7.

† Hyperglycemia persisted in spite of the daily administration of these doses of insulin. More than one value is listed if the dose was changed greatly.

‡ On March 22, 1958, 400 units of crystalline insulin produced intermittent hypoglycemia which lasted for 40 hours.

temporarily overcome by the administration of a sufficiently large dosage of insulin; indeed this was accomplished in Case 4 by the administration (on March 22, 1958) of 400 units of crystalline insulin over a period of six hours. Following this large dose of insulin there was intermittent hypoglycemia for forty hours. Berson and Yalow (20) have noted that manifestations of hypoglycemia may persist for several days in insulin-resistant subjects following a large dose of insulin.

Moloney and Aprile (24) showed that insulin mixed with anti-insulin at pH 7.0–8.0 (from insulin-treated guinea pigs or horses) could be precipitated at −5° C. by alcohol (25 per cent v/v). This precipitated insulin could then be extracted by alcohol (70 per cent v/v, pH 2.5). Insulin mixed with normal serum from the guinea pig or horse was not precipitated by alcohol (25 per cent v/v) at −5° C.

Analogously, insulin mixed with the serum of the patient was found to be precipitated in the cold by alcohol (25 per cent v/v), whereas insulin with normal serum was not so precipitated. Results of the precipitation of mixtures of insulin and serum from a number of subjects is shown in Table 6.

It is clear from the data in the table that insulin mixed with serum from Case 1 was precipitated in the cold by alcohol (25 per cent), whereas insulin mixed with normal serum or with serum from a diabetic who was not receiving insulin was not precipitated under these conditions. Results

TABLE 6. PRECIPITATION OF INSULIN-SERUM WITH ALCOHOL (25% at −5°C.)

Subject	Daily insulin requirement (units)	Mixture pptd.		Insulin (units) recovered by extraction with acid alcohol from	
		Serum (ml.)	Insulin (units)	Supernatant	Precipitate
Diabetic (Case 1)	>1,000	10	4	<1.0	1.0*
Diabetic†	0	5	1	1.0	<1.0
Diabetic†	18 PZI	5	1	<1.0	<1.0
Diabetic†	12 PZI	5	1	<1.0	<1.0
Normal	0	10	4	2–4	<0.5
Normal	0	10	2	2	<0.5
Normal	0	10	2	2	<0.5

* Extraction of insulin+anti-insulin complex does not yield 100%.
† Case not otherwise dealt with in this paper.

were equivocal with the sera from the 2 diabetics who were receiving moderate amounts of insulin.

Serum from Case 1 was fractionated by precipitation with ammonium sulphate. The fraction precipitated by the addition of 17.5 Gm. of ammonium sulphate per 100 ml. of diluted serum was discarded; the neutralizing factor in the supernatant (as measured by inhibition of insulin-induced convulsions in mice) was precipitated by increasing the concentration of ammonium sulphate to 28.0 Gm. per 100 ml.

DISCUSSION

The question whether or not there are neutralizing antibodies to insulin is still being asked and must be considered. LaPresle and Grabar (25) and Scheiffarth, Frenger and Möckel (26), using tanned red-cell technique, have shown that antibodies which might be ascribed to insulin may bear no relation to it. Hence the results of any *in vitro* test for insulin-neutralizing antibodies must be regarded with caution. The question of antigen or antigens as a cause of allergy also presents itself. Allergy to insulin some-

times occurs without clinical insulin resistance, or resistance may be present without clinical evidence of allergy. In the present series 2 of the cases demonstrated both resistance to insulin and allergy; in the one case the allergy manifested itself by urticaria, anaphylaxis and eosinophilia; in the other, by urticaria. Since crystalline insulin often contains active antigen (25) other than insulin, it follows that various manifestations of allergy may be due either to contaminant or to insulin itself. In this case both antibodies may have been induced by insulin, or one by insulin and one by contaminant in the crystalline insulin.

Direct evidence for the existence of neutralizing antibodies was obtained by Moloney and Aprile (24) from a study of the serum of a horse which had been given a series of injections of crystalline ox insulin. At a certain stage in the course of inoculation, serum specimens possessed insulin-neutralizing property (inhibition of insulin-induced convulsions in mice); at a later stage, not only was insulin neutralized but also flocculation was produced when the serum was mixed with insulin. Furthermore, from washed floccules, both insulin and neutralizing anti-insulin could be obtained in good yield. It may be concluded, then, that neutralization and flocculation of insulin by the serum specimens which exhibited both these properties, was due to one and the same antibody.

Since an antibody accounted for both neutralization and flocculation, it is therefore most probable that neutralization of insulin by serum specimens which were taken earlier in the course of immunization of the horse and did not exhibit flocculation, was due also to a specific antibody. The validity of this assumption is strengthened by the observation of Moloney and Aprile (27) that insulin mixed with neutralizing but nonflocculating antiserum (horse) is precipitated in the cold by ethanol (25 per cent, v/v), whereas insulin mixed with normal horse serum is not, and that insulin can be recovered from the precipitate by extraction with acid alcohol, just as with floccules.

In Case 1 presented here, it is most probable also that neutralization of insulin shown by the patient's serum was due to antibody, for the following reasons: The property of neutralization appeared in the serum following a series of injections of the protein, insulin; the neutralizing property was associated with the globulin fraction of the serum (ammonium sulphate fractionation); insulin could be recovered from a neutral insulin + anti-insulin mixtures; alcohol in a concentration of 25 per cent did not in the cold precipitate insulin mixed with normal serum (pH of mixture, approx. 7.5) but did precipitate insulin mixed with neutralizing serum from the patient; and from the alcohol precipitate insulin could be recovered by extraction with acid alcohol. Furthermore, the pattern of

neutralization of insulin by the serum from Case 1 was identical with that shown by the sera of animals bearing anti-insulin *i.e.*, the serum of the patient neutralized all the extracted insulins tested, including that of the human subject but not of the guinea pig.

Lowell (28) reported 2 cases of diabetes with resistance to commercial insulin (ox or pig). One responded to human insulin and the other did not. Serum from the responsive case did not neutralize human insulin (inhibition of insulin-induced convulsions in mice), whereas serum from the unresponsive case did. Sherman (9) also reported a patient whose serum neutralized commercial insulin but not human insulin. Thus, of the sera of 4 diabetics who were resistant to insulin, 2 neutralized human insulin and 2 did not. These differences among antisera in respect to neutralization of *extracted* human insulin may depend upon quantitative differences in avidity of the sera or, as suggested by Lowell, upon qualitative differences of specificity. For Case 1, there is good evidence that the patient's *endogenous* insulin was not neutralized by the antibodies to insulin which she was carrying, since the diabetes was controlled by diet alone.

Extracted guinea-pig insulin differs chemically from ox insulin (29). Moreover, as shown by the mouse-convulsion test, guinea-pig insulin is not neutralized by antibodies induced in various species of animals. In contrast to this observation, it has been found (28) that extracted guinea-pig insulin fails to lower the blood sugar level of a guinea pig bearing induced anti-insulin, when injected in an amount effective in a normal guinea pig. It is unlikely, therefore, that extracted guinea-pig insulin would be useful in the treatment of patients with neutralizing antibodies.

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TREATMENT OF OVARIAN DYSFUNCTION WITH SMALL DOSES OF CORTISONE OR HYDROCORTISONE*

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ABSTRACT

Because mild hirsutism or a high level of urinary 17-ketosteroid excretion is often observed in women with functional irregularities of menstruation, the possibility exists that there may be an associated mild disorder of adrenocortical function. If such were the case, treatment with suitable doses of cortisone or hydrocortisone might be beneficial. Encouraging results were obtained with oral administration of either of these steroids in doses of 2.5 to 5.0 mg. at intervals of eight hours or less to women with hirsutism and infertility. This led to an extension of the trials to include any patients with irregularity or absence of the menses on a functional basis. Among 52 such women, therapy resulted in resumption or increased regularity of ovulation in 46 (89 per cent). Of 38 patients in the series who had associated infertility, 21 (55 per cent) had one or more pregnancies while receiving this type of treatment. No untoward effects were observed. In some instances, hirsutism, acne, or chronic cystic mastitis also improved. The results suggest that an appreciable number of cases of ovarian dysfunction may be related to mild disorders of adrenocortical function, and indicate that continual low-dosage adrenocortical steroid therapy is a practical and effective treatment for such patients.

FUNCTIONAL disorders of the ovaries have been attributed to many causes, but evidence for the relationship between cause and effect has been largely indirect or speculative. One of the most common types of ovarian dysfunction is characterized by irregularity of the menstrual cycle. This may begin at the time of menarche, or develop after several years of regular menses. Ovulations may occur with each irregular cycle, once every several cycles, or not at all. The irregularity of the cycles may progress to such a degree that menses occur only once or twice a year, or they may cease altogether, producing the condition of secondary amenorrhea. Stein and Leventhal (1–3) called attention to a group of patients with these symptoms who also had enlarged, polycystic ovaries. Approximately half

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of their patients had excessive hair growth on the face or body, and 10 per cent were obese, so they postulated that the ovarian dysfunction was caused by an endocrine disorder of unknown nature. The occurrence of enlarged polycystic ovaries is relatively uncommon, however, so the Stein-Leventhal syndrome in its strict sense accounts for a rather small percentage of patients with symptoms of this type.

In the female, a possible cause of excessive hair growth is an increased production of androgen by the adrenal cortex. This might be associated with an elevation of the urinary excretion of 17-ketosteroids. When it was observed that many women with irregular menses or secondary amenorrhea, regardless of the presence of enlarged polycystic ovaries, had excessive hair growth or high urinary 17-ketosteroid excretion, or both, the possibility that they might have an underlying disorder of adrenocortical function was therefore considered. Such a disorder might be a mild variant of the syndrome of congenital adrenal hyperplasia, a type of adrenal dysfunction that has been found to respond favorably to the administration of suitable doses of cortisone or hydrocortisone (4).

Cortisone therapy in patients with ovarian dysfunction accompanying hirsutism has been tried by others (5–10). However, the daily dosage, at least initially, was 50 mg. or more—a level that might produce complete suppression of the adrenal cortices, or even hypercorticism. This not only raised a question of whether the favorable responses were due to pharmacologic rather than physiologic effects of the steroid, but also caused some reluctance to administer such doses to otherwise healthy women.

In this clinic, after trials with numerous dosages and schedules of therapy, much smaller amounts of steroid given at intervals of eight hours or less were found to produce beneficial results. Initially, patients with associated infertility were treated (11). The encouraging results with small doses and the absence of untoward effects led to an extension of therapeutic trials to any patient with irregularity or absence of menses of a functional type. The data, which suggest that a larger number of cases of ovarian dysfunction may be related to disorders of adrenocortical function than has hitherto been suspected, form the basis of this report.

METHODS

The 52 women included in this study varied in age from 17 to 36 years. All underwent careful examinations and appropriate tests to rule out the presence of tumor or other known causes of ovarian dysfunction. Many were known to be infertile. Practically all had previously received various types of therapy, including thyroid and cyclic estrogen and progesterone, without improvement, although most had experienced withdrawal bleeding after administration of progesterone. In some, the menstrual disorder had progressed to a state of amenorrhea of several years' duration. Increase in hair growth,

when present, varied from a slight excess (especially in the periareolar and subumbilical areas) to moderately severe hirsutism of the face and body. There were no masculinizing changes of the voice in any patients, and enlargement of the clitoris was rarely seen.

Careful records were kept of the basal body temperatures of these women, and ovulation was detected by the characteristic spontaneous rise of temperature followed by menses or pregnancy. Women with irregular menses were classified as having increased frequency of ovulation when they had 3 successive ovulatory cycles of thirty-five days or less.

Urinary 17-ketosteroids were determined as follows[1]: A 5-ml. aliquot of urine, 10 ml. of water, 0.1 ml. of 8 per cent formaldehyde and 6 ml. of concentrated hydrochloric acid were heated in a boiling-water bath for ten minutes, cooled, and extracted once with 20 ml. of ethylene dichloride. After washing with 5 ml. of 3 *N* sodium hydroxide and water to neutral, a 10-ml. portion of the extract was evaporated to dryness and the Zimmermann color reaction carried out by the method of Wilson and Carter (12), except that the alcohol, meta-dinitrobenzene and alcoholic potassium hydroxide were mixed just prior to adding 0.4 ml. to each tube. The color correction of Talbot *et al.* (13) was routinely employed. Each analysis was performed in duplicate and required to check within 5 per cent. In order to determine the reproducibility of this method as employed in our laboratory, 6 or more complete assays were run on each of a series of 24-hour urine specimens on different days, totaling 102 assays on urines of which the mean 17-ketosteroid content ranged from 2.4 to 22.2 mg. per twenty-four hours. The standard error for the entire group was 1.17 mg., and the standard deviation for each specimen did not exceed 2.3 mg. The verification of the ketonic nature of the material measured was accomplished through the use of a Girard separation on representative samples.

In a series of 31 healthy women with regular menses and no hirsutism, aged 18 to 42 years, the mean 24-hour urinary 17-ketosteroid excretion measured by this method was 9.5 mg., with a standard deviation of 2.9 mg., and a range of 5.2 to 19.0 mg. In only 3 subjects of the group did the values exceed 13 mg. For the purpose of classification, 24-hour 17-ketosteroid excretions greater than 13 mg. were arbitrarily designated as "high" levels of excretion.

The usual procedure was to obtain at least two 24-hour urine collections for 17-ketosteroid determinations before therapy, and to continue these determinations at monthly intervals during the course of treatment.

Cortisone acetate[2] or hydrocortisone[3] tablets were administered orally. The dosage employed was the smallest that would reduce the high urinary 17-ketosteroid excretion to 10 mg. per day or less, when the schedule was either every eight hours or four times daily. With the latter schedule, a dose was taken at each mealtime and one at bedtime. The effective dosage was found to vary with individual patients; it ranged from 2.5 to 5.0 mg. four times daily, the most frequently effective dosage being 5.0 mg. every eight hours. For patients with normal 17-ketosteroid excretion prior to therapy, the initial dosage was usually 5 mg. every eight hours; if no improvement had occurred after three months, it was reduced to 2.5 mg. four times daily.

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[1] We are indebted to Dr. J. R. Leonards for helpful suggestions and assistance in the development and standardization of this method in our laboratory.

[2] Cortone acetate, generously supplied by Drs. Elmer Alpert and F. R. Heath, of the Merck Sharp & Dohme Laboratories.

[3] Cortef, generously supplied by Drs. Harold L. Upjohn and H. C. Peltier, of the Upjohn Company.

RESULTS

Depending upon the presence of increased hair growth or a "high" urinary excretion of 17-ketosteroids, the patients were classified as follows:

Group A: Excessive hair and high 17-ketosteroid excretion

The results in this group are listed in Tables 1 and 2. In all 26 cases, 17-ketosteroid excretion returned to normal (10 mg. or less on repeated determinations) within two months after starting the effective dose of steroid.

TABLE 1. EFFECTS OF LOW-DOSAGE ADRENOCORTICOSTEROID THERAPY IN OVARIAN DYSFUNCTION

	Group A*	Group B*	Group C*	Group D*	Total
Total number of patients	26	14	2	10	52
Restoration or increased frequency of ovulatory menses	23 (89%)	13 (93%)	2	8 (80%)	46 (89%)
Fertility problems	13	14	2	9	38
Patients who became pregnant	7 (54%)	9 (64%)	2	3 (33%)	21 (55%)
Miscarriages	0	1†	1‡	0	2
Full-term deliveries	6	6	1	1	14
Pregnant	1	3	0	2	6

* For classification, see text.

† This patient subsequently became pregnant again and carried the fetus to term uneventfully (see text).

‡ This patient has a small nontoxic diffuse goiter and is receiving both thyroid and cortisone therapy (see text).

Seven patients had pre-treatment 17-ketosteroid excretions of 20 mg. or more per day; in 1 the excretion reached levels of 32.0 and 32.6 mg. on separate occasions. In 23 patients there was restoration of, or increased frequency of ovulatory menses one to six months (usually three months) after starting the effective dose. In 10 patients the initial regimen was effective, but in the remaining 13 at least one change in steroid dosage was necessary in order to bring the 17-ketosteroid excretion within the normal range. The most frequently effective dosages were 5 mg. every eight hours (13 patients) and 5 mg. four times daily (7 patients). Two patients responded to 2.5 mg. four times daily, and one required a total of 25 mg. daily to bring the 17-ketosteroid excretion to normal and restore ovulation. In the 3 patients who did not experience improvement in ovarian function, 17-ketosteroid excretion was maintained in the normal range by steroid treatment for at least six months.

Of the 13 women with infertility problems, 7 became pregnant from two

TABLE 2. EFFECT OF LOW-DOSAGE ADRENOCORTICOSTEROID THERAPY IN OVARIAN DYSFUNCTION ASSOCIATED WITH HIRSUTISM AND HIGH URINARY 17-KETOSTEROID EXCRETION

Patient	17-Ketosteroids (mg./24 hrs.)		Restoration of ovulation	Effective dosage of steroid†	Duration of effective therapy‡	Fertility problem	Pregnancy
	Before treatment*	During treatment					
1	18.8	8.9	Yes	F, 5 mg. q. 8 h.	3 mos.		
2	14.3	6.3	Yes	F, 5 mg. q. 8 h.	3 mos.		
3	16.3	§	Yes	F, 5 mg. q. 8 h.	5 mos.		
4	24.2	7.5	Yes	F, 5 mg. q. 8 h.	6 mos.		
5	19.3	9.3	Yes	F, 5 mg. q. 8 h.	2 mos.	Yes	Yes
6	16.5	7.1	Yes	F, 5 mg. q. 8 h.	2 mos.	Yes	
7	14.5	6.3	Yes	F, 5 mg. q. 8 h.	3 mos.		
8	19.8	8.3	Yes	F, 5 mg. q. 8 h.	6 mos.	Yes	
9	13.4	10.0	Yes	F, 5 mg. q. 8 h.	3 mos.	Yes	
10	19.0	8.4	Yes	F, 5 mg. q. 8 h.	6 mos.		
11	19.8	9.4	No	F, 5 mg. q. 8 h.‖		Yes	
12	16.1	10.0	No	F, 5 mg. q. 8 h.‖			
13	21.2	9.1	No	F, 5 mg. q. 8 h.‖		Yes	
14	14.3	10.4	Yes	E, 5 mg. q. 8 h.	2 mos.	Yes	Yes
15	14.0	8.7	Yes	E, 5 mg. q. 8 h.	5 wks.	Yes	Yes
16	20.0	9.4	Yes	E, 5 mg. q. 8 h.	3 mos.		
17	24.0	5.6	Yes	F, 5 mg. 4×d.	3 mos		
18	19.8	5.4	Yes	F, 5 mg. 4×d.	5 mos.		
19	20.4	8.0	Yes	F, 5 mg. 4×d.	6 wks.	Yes	Yes
20	13.9	10.4	Yes	F, 5 mg. 4×d.	1 mo.		
21	32.3	10.0	Yes	F, 5 mg. 4×d.	1 mo.	Yes	Yes
22	19.0	9.3	Yes	E, 5 mg. 4×d.	6 wks.	Yes	Yes
23	24.4	9.7	Yes	E, 5 mg. 4×d.	1 mo.	Yes	
24	19.4	10.0	Yes	F, 25 mg./d.	1 mo.		
25	15.5	9.3	Yes	F, 2.5 mg. 4×d.	2 mos.	Yes	Yes
26	14.1	6.5	Yes	F, 2.5 mg. 4×d.	4 mos.		

* Average value.

† Abbreviations: F = hydrocortisone; E = cortisone acetate; q. 8 h. = every 8 hours; 4×d. = 4 times daily.

‡ At time of restoration of regular ovulation or conception.

§ Not available.

‖ Effective in reducing 17-ketosteroid excretion to normal, but not in restoring ovulation.

to six months after starting the effective dose. Six of these patients had full-term normal deliveries and one is pregnant.

Four of the women in this group had amenorrhea of over one year's duration at the time therapy was initiated. In 2 of these patients ovulation was resumed during treatment, and 1 of the 2 (with pre-treatment 17-ketosteroids of 32 mg.) became pregnant and had a full-term normal delivery. The other was not married.

Group B: Excessive hair, but normal 17-ketosteroid excretion

In 13 of the 14 women in this group (Tables 1 and 3) there was restoration of, or increased frequency of ovulatory menses during therapy within two weeks to five months, usually within three months. Seven patients responded to a dosage of 5 mg. every eight hours, and 5 responded to 2.5 mg. four times daily; only 1 experienced more regular ovulations during

TABLE 3. EFFECT OF LOW-DOSAGE ADRENOCORTICOSTEROID THERAPY IN OVARIAN DYSFUNCTION ASSOCIATED WITH HIRSUTISM AND NORMAL URINARY 17-KETOSTEROID EXCRETION

Patient	17-Ketosteroids (mg./24 hrs.)		Restoration of ovulation	Effective dosage of steroid†	Duration of effective therapy‡	Fertility problem	Pregnancy
	Before treatment*	During treatment					
1	12.9	8.5	Yes	F, 5 mg. q. 8 h.	1 mo.	Yes	Yes
2	11.6	8.0	Yes	F, 5 mg. q. 8 h.	5 mos.	Yes	Yes
3	5.0	4.9	Yes	F, 5 mg. q. 8 h.	2 mos.	Yes	
4	8.2	7.9	Yes	E, 5 mg. q. 8 h.	6 wks.	Yes	Yes
5	7.8	6.7	Yes	E, 5 mg. q. 8 h.	5 wks.	Yes	Yes
6	6.1	5.9	Yes	E, 5 mg. q. 8 h.	3 mos.	Yes	Yes
7	3.2	3.2	Yes	E, 5 mg. q. 8 h.	3 mos.	Yes	
8	6.7	§	Yes	F, 2.5 mg. 4×d.	3 mos.	Yes	Yes
9	11.0	7.3	Yes	F, 2.5 mg. 4×d.	2 wks.	Yes	Yes
10	9.5	4.1	Yes	F, 2.5 mg. 4×d.	2 mos.	Yes	
11	6.5	7.0	Yes	E, 2.5 mg. 4×d.	1 mo.	Yes	Yes
12	9.1	§	Yes	E, 2.5 mg. 4×d.	6 wks.	Yes	Yes
13	12.3	7.5	Yes	E, 25 mg./d.	3 mos.	Yes	
14	11.9	6.3	No	‖		Yes	

* Average value.

† Abbreviations as in Table 2.

‡ At time of restoration of regular ovulation or conception.

§ Not available.

‖ This patient received cortisone, 5 mg. every 8 hours, for 6 months without benefit. She then moved to another city.

treatment with 25 mg. daily than with smaller doses. All 14 patients in this group had infertility problems, but 9 became pregnant during therapy. One patient (1) had a miscarriage in the fifth month of pregnancy. Because she had a small diffuse nontoxic goiter, sodium-*l*-thyroxine[4] (0.2 mg. daily) was added to the hydrocortisone, which was continued at the same dosage level. She conceived again two months later and had a full-term normal delivery. Desiccated thyroid (0.2 Gm. daily) had been taken prior to steroid therapy for over a year without improvement. Five of the other pregnant women had full-term normal deliveries, and the other 3 are pregnant.

Group C: Hair not excessive, but high 17-ketosteroid excretion

Only 2 women were in this group (Table 1). The 17-ketosteroid excretion of both patients decreased to normal during corticosteroid treatment. They became pregnant, but one miscarried a month after conception. This patient also has a small nontoxic diffuse goiter, so a mild disturbance of thyroid function may have been a factor in the miscarriage. The other patient had a full-term normal delivery. Her pre-treatment 24-hour 17-ketosteroid excretion was 20.6 mg., and the dosage of cortisone was 5 mg. four times daily.

Group D: Hair not excessive, and 17-ketosteroid excretion normal

There were 10 women in this group (Table 1). As these patients presented no stigmata suggestive of adrenal disturbance, the only reason for including them in the study was the presence of functional irregularity of the menses or secondary amenorrhea. Yet in 8 of these patients there was restoration or increased frequency of ovulatory menses in from two weeks to four months after initiation of corticosteroid therapy. The effective dosages were 5 mg. every eight hours in 4 patients, and 2.5 mg. four times daily in 4. Of the 9 women with infertility problems only 3 conceived; 1 had a normal delivery and the other 2 are pregnant.

General observations

In 2 patients with moderately severe hirsutism there was a definite improvement in hair growth after six months of therapy, the hair becoming softer and finer in texture. One woman was able to stop shaving her face altogether, and the other had to shave only once a week instead of every other day. Most of the patients with excess hair noted little change, however. Because of the long cycle of hair growth, it is possible that improvement might have occurred with longer periods of therapy. Hypertrichosis

[4] Synthroid, generously supplied by Travenol Laboratories.

resulting from adrenal tumors, for example, may persist for over a year after removal of the tumor.

Many of the women had mild to moderate acne, and most of them noted distinct improvement within two months after starting therapy.

A few patients had chronic cystic mastitis of mild to moderate degree. This condition subsided during steroid therapy. Such changes may have been coincidental, but it is possible that correction of ovarian dysfunction as manifested by return of ovulatory cycles may have been causally related. These observations warrant further study.

Side-effects of corticosteroid therapy

No untoward symptoms developed as a result of therapy with cortisone or hydrocortisone at these lower levels of dosage. Furthermore, there was no indication that this type of steroid therapy impaired the response to stress. Several patients had Asian-type influenza during the recent epidemic, while they were receiving corticosteroids; without any change in dosage they recovered normally without complications. One woman underwent a hemithyroidectomy for a nontoxic thyroid nodule in the fifth month of pregnancy, while receiving hydrocortisone (5 mg. every eight hours). Another underwent cesarean section at term, while receiving cortisone in the same dosage. Both women had normal operative and postoperative courses without need for an increase in steroid dosage. Moreover, patients who received continual steroid therapy for periods as long as two years had no untoward symptoms upon cessation of treatment.

Because of the suggestive evidence of an underlying disorder of adrenal function, women who became pregnant were given corticosteroids in the same dosage throughout their pregnancies. Eleven of the 14 babies delivered were normal in every respect. Three infants whose mothers had known blood-type incompatibilities and had had previous pregnancies, suffered from erythroblastosis fetalis, but they responded to exchange transfusions and are now well. Of the 2 women who had miscarriages while receiving steroid treatment, one subsequently had a term pregnancy during which she received thyroid therapy in addition to steroids. These results contrast with the relatively high incidence of miscarriages (almost 50 per cent) reported in patients of this type who became pregnant while taking larger doses of cortisone and whose treatment was withdrawn early in the pregnancy (6, 9). The data suggest that continuation of low-dosage corticosteroid therapy may help prevent miscarriages in some patients.

Duration of treatment

If the underlying disorder in these women is a mild defect in adreno-

cortical function, one might postulate that corticosteroid treatment should be continued indefinitely. In an attempt to answer this question, steroid therapy was stopped after delivery in patients who had term pregnancies. So far only 5 women have been observed for a sufficient period of time after withdrawal to evaluate their response. In 3 whose treatment was stopped immediately post partum, there was a recurrence of ovarian dysfunction, and they have requested resumption of medication. In the other 2, steroid therapy was continued post partum until regular ovulatory cycles were resumed; after 3 ovulations, medication was discontinued. One conceived with an ovulation that occurred six days after cessation of therapy. The other woman's cycles became more irregular, but she also became pregnant again three months after cessation of therapy. It is evident that at least in some women there will be a recurrence of ovarian dysfunction if steroid treatment is terminated. Whether a remission can be maintained under these circumstances remains to be determined.

Studies of adrenocortical function

Studies to determine the nature of the possible disorder of adrenocortical function in the several groups of patients are still in progress. The responses of representative patients with either high or normal 17-ketosteroid excretion to ACTH stimulation did not differ significantly from that of a normal subject of the same age group with respect to sodium retention or potassium loss. In some there was a normal rise in total 17-ketosteroid excretion; in others there was no significant change.

If these cases represent mild variants of congenital adrenal hyperplasia, a disorder that is apparently caused by a defect in hydroxylation of C_{21} of the steroid molecule (14), an increased excretion of pregnanetriol would be expected. The nonketonic fraction obtained from the urine of 2 patients in this series with high 17-ketosteroid excretions was examined by Kewish (15), using the method of de Courcy (16) for the detection of Δ^5-pregnene-3β,17α,20αtriol (I) and pregnane-3α,17α,20α-triol (II), and adapted for quantitative measurements. In one case he found 2–3 mg. of I and 2–3 mg. of II per day; in the other he found 1–2 mg. of I and only a trace of II. The urine of a normal woman contained 0.5 mg. of I and 1.0 mg. of II per day. These values may indicate that the levels of I are slightly elevated, but data on a much larger series of patients and controls are required before the significance of these figures can be established.

A number of investigators have reported that at least some hirsute women with or without the Stein-Leventhal syndrome excrete an excess of certain fractions of urinary 17-ketosteroids (17–22), but differences in technics of hydrolysis and fractionation make a comparison of the values for specific fractions difficult. Studies to determine the pattern of adrenal

steroid excretion in the 4 groups of patients included in this report, both prior to and during corticosteroid therapy, are in progress.

DISCUSSION

Evidence is accumulating rapidly that the clinical picture known as "idiopathic hirsutism" in females is associated with a relatively mild disorder of steroid production. This possibility has been considered for many years; Luft (23) and Bissell and Williams (24) commented upon it in their excellent reviews fourteen years ago. Total 17-ketosteroid excretion was frequently, but not always, elevated in individual cases. The use of improved technics of hydrolysis and fractionation of urinary steroids resulted in reports of abnormal patterns in representative women. Such patients often had associated disturbances in ovarian function.

Although the adrenal cortex would seem to be the more likely source of the abnormal steroid production, the favorable response of ovarian function to wedge resection of the ovaries and the occurrence of certain histologic changes in the ovaries (3, 25) suggest the alternate possibility that the ovaries might be the site of the abnormal steroid metabolism.

The observation that ovarian dysfunction in patients of this type may respond to small doses of cortisone or hydrocortisone administered at frequent intervals provides additional evidence favoring the concept that the underlying disorder in such cases has its origin in the adrenal cortex. The dosage of these steroids given in our series of patients was considerably less than the 40 to 50 mg. daily required to maintain an adrenalectomized patient, and hence could hardly provide an excessive supply of adrenal hormone to the tissues. However, the restoration of normal levels of 17-ketosteroid excretion in patients whose pre-treatment levels were high, clearly indicates that the therapy caused a change in steroid production. It seems likely, therefore, that the small dose inhibits an excessive adrenocortical activity that has resulted from a relative defect in one of the enzyme systems necessary for the production of hydrocortisone. In order to supply sufficient hydrocortisone for bodily needs, such defective adrenals probably are stimulated to an increased rate of activity, and in the process manufacture excesses of certain steroids that are the cause of the androgenic effects or the interference with ovarian function. Proof of this hypothesis must await actual demonstration of the pattern of adrenal steroid production before and during therapy. This is especially pertinent with regard to the patients whose total 17-ketosteroid excretion was within normal limits, because the only evidence of steroid abnormality was their clinical response to treatment.

The observation that relatively mild disorders of steroid production may be present in women with hirsutism or ovarian dysfunction requires a

reappraisal of normal values for 17-ketosteroid excretion. Unless women with any degree of excessive hair growth or ovarian dysfunction are omitted, a series cannot be considered to be normal. In previously published series there have been no reports of such screening, so it is possible that this factor may have caused a greater spread in the presumed normal range, especially with respect to the higher values.

The possibility that mild disorders of adrenocortical function may occur without the characteristic clinical picture of the adrenogenital syndrome or Cushing's syndrome also suggests that a more far-reaching reappraisal of the role of the adrenal cortex in other diseases is advisable. Heretofore, the absence of gross clinical evidence of adrenocortical disturbance and a normal response of circulating eosinophils, urinary 17-ketosteroids and urinary 17-hydroxycorticosteroids to ACTH has been considered sufficient to exclude a diagnosis of adrenal dysfunction. These criteria are no longer adequate, and until the exact pattern of steroid production is demonstrated, the possibility that mild adrenal disorders may be contributing factors in the etiology of many diseases, especially those precipitated or aggravated by stress, cannot be excluded.

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METABOLIC OBSERVATIONS IN A CASE OF PURE PRIMARY HYPERALDOSTERONISM

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ABSTRACT

Steroidal and metabolic studies were carried out in a typical case of pure primary hyperaldosteronism. Analysis of the urine for known steroids failed to show significant abnormalities other than hyperaldosteronuria and a low excretion of 5α-pregnane-3β,16α-diol-20-one (sodium-excreting factor). ACTH stimulated the production of corticosteroids, including aldosterone, and 9α-fluoro-Δ^1-cortisol inhibited production. Extract of the posterior hypophysis caused an increase in aldosterone excretion without altering that of other steroids. The effect of sodium excess on aldosterone was studied. The data indicate that production of aldosterone in this case was not independent of controlling hormonal and humoral factors. Metabolic studies demonstrated, 1) an approximate parallelism between sodium and potassium balances, 2) rapid natriuresis of a sodium load, and 3) lack of correlation between aldosteronuria and sodium balance. The physiopathologic implications of these facts are discussed.

CONN (1) in 1955 described as a case of primary aldosteronism a well studied syndrome in which arterial hypertension, hypokaliemia and alkalosis were the major clinical features. The lack of edema was an important negative finding. The discovery of hyperaldosteronuria without 17-ketosteroid or 17-hydroxysteroid abnormalities, the existence of an adenoma (aldosteronoma), and a salvaging surgical cure strongly supported his assumption.

In a typical case (2), our own investigations were designed to contribute towards the elucidation of the following current problems:

1. What is the total steroidal pattern of this syndrome as studied by analytical methods?

No *in vivo* steroid studies have been made except those of Garrod, Ayres and associates (3, 4). In 3 cases they found that aldosterone levels in the plasma and urine were either normal or only slightly elevated, and suggested that the aldosterone level might be high by comparison with that in other hypokaliemic subjects (5). In addition they found a normal excretion of corticosterone in 2 patients with tumors and an elevated excretion

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in a third. They did not find an excess of cortisol, nor did they detect the presence of any other "electrolyte active factor." Recently Neher (6) in 3 adrenal tumors studied *in vitro* found elevated aldosterone and normal cortisol-cortisone levels; in 1 case there was an excess of corticosterone.

Thus in primary aldosteronism, hyperaldosteronism is often an isolated finding. However, in some cases there is hypercorticosteronism, as was first suggested by Mader and Iseri (7). This may account for the elevated "blue tetrazolium urinary corticoids," but the biologic properties of corticosterone leave unexplained the metabolic findings in the syndrome described by Conn *et al.* (8).

In the present paper, the individual urinary metabolites of the adrenal hormones in a typical case are reported.

2. Is the adenoma autonomous, or is its secretion(s) subject to control(s) by some hormonal or humoral changes in the body? This point has not been extensively studied.

In Conn's report, a normal response of 17-hydroxycorticoids and 17-ketosteroids to administration of ACTH was described, but increased aldosterone production was only deduced on the basis of sodium retention after ACTH (1). In the observations of Garrod *et al.* (3), mention was made of the lack of increase of aldosterone during administration of potassium.

In the present paper, observations are reported on aldosterone and other corticosteroids after administration of ACTH, posterior pituitary extract, 9α-fluoro-Δ^1-cortisol, and a sodium chloride load.

3. What correlation may be drawn between these steroid data, especially the day-to-day aldosterone measurements, and the data from a metabolic balance study? No such correlative work has been reported to date.

CASE REPORT[1]

Mrs. G. was a 45-year-old housewife with longstanding arterial hypertension, known for the previous fourteen years. Her weight was 119 pounds (54 Kg.) and her height was 5 feet 1 inch (154 cm.) Cardiac and ocular symptoms were absent or minimal, and edema had never been observed. For the previous seven years she had suffered from chronic asthenia, periodic paretic crises, and tetany manifested by recurrent episodes of carpopedal spasms. Polydipsia with polyuria (average, 1900 ml. of urine per day) and albuminuria (average, 0.75 Gm. per day) were quite constant at the time of hospitalization. Hyposthenuria was not modified by restriction of water or administration of posterior hypophyseal extract.

Hypokaliemia was constant, the mean concentration of potassium being 2.4 mEq. per liter, with a range of 2.1 to 2.9 mEq.; electrocardiographic findings were compatible with these levels. The concentration of potassium in the saliva was elevated (44 mEq. per liter versus a normal value of 23 mEq.) and urinary excretion was relatively increased (30 mEq. per day). The plasma sodium level was stable (140 mEq. ± 3 per liter) and the chloride level was 100 mEq. ± 3 per liter. Plasma CO_2 combining power aver-

[1] For a more detailed clinical report, see (2).

aged 30 mEq. per liter, with a range of 26.4 to 33.7 mEq. The pH of the urine was always greater than 6.5, more often greater than 7.5.

The blood urea level was normal, with an urea clearance of 73 ml. (maximal flow formula); by contrast, the glomerular filtration rate (mannitol) was 60 ml. and the renal blood flow (p-aminohippuric acid test) was 380 ml. (day 40).

Other abnormal laboratory findings were: a distinct hypochromic microcytic anemia (red blood cell count 3,600,000 per cu. mm.); mild bruisability with some capillary fragility; a moderate increase in concentration of the main α_2-mucoprotein (haptoglobin); a flat glucose tolerance curve with delayed return to normal; an elevated level of serum aldolase (1.3 U. per liter versus the (normal 0.25 U.); hypomagnesemia (1.1 mEq. per liter) with normal urinary excretion of magnesium; and constant hypocalciuria associated with normal levels of serum calcium and phosphorus and partial retention of calcium after an intravenous calcium load test.

Hyperaldosteronuria was the only steroidal abnormality found by routine determinations.

X-ray examination by tomography after presacral insufflation of oxygen revealed a left adrenal mass.

The *operation* was performed[2] by the abdominal approach without preoperative hormonal therapy. The right adrenal appeared slightly decreased in size although subsequent histologic examination of the biopsy specimens showed it to be normal. In the left adrenal gland a yellow adenoma (7 Gm.) composed of large spongy cells of the fascicular type was surrounded by adrenal tissue that was normal in appearance and histology. Left kidney biopsy showed diffuse vacular lesions in the proximal convoluted tubules and diffuse fibrinoid changes in the arteriolar walls. Some focal lesions of nephron atrophy by interstitial sclerosis were noted.[3]

Postoperative course: In the first ten days the electrolyte levels and ECG tracings returned concurrently to normal; the tetany vanished; and, as the polydipsia disappeared, the urine volume fell to 800 ml. per day (without proteinuria). The anemia disappeared in the fourth month. The blood urea nitrogen level, however, increased to 50 mg. per 100 ml. during the first three months, and on the thirty-seventh postoperative day the urea clearance was 20 ml. per minute (maximum flow formula). Eight months after adenomectomy the blood urea nitrogen value was 35 mg. per 100 ml. and the hypertension had been partially ameliorated. Urinary aldosterone excretion, which had been followed until the sixth month, remained low (less than 1 μg.), but the other corticosteroids were normal. With the exception of a mild episode of thrombophlebitis in the right leg, the postoperative course was uneventful.

METHODS

Clinical management

In periods II to VI inclusive, balance studies (Fig. 1) were carried out for sodium, potassium and water; a constant caloric and nitrogen intake was maintained. Only the urinary constituents were determined, except for one 3-day period when fecal potassium was measured. We have arbitrarily added 4 mEq. of sodium per day and 15 mEq. of potassium per day for the fecal excretion (indicated in black in Figure 1).

A basal dietary Na-K ratio was chosen in order to reduce, if possible, the hyperaldosteronism, *i.e.*, a relatively high sodium intake (averaging 180 mEq. per day) and a low

[2] By Prof. C. Dubost.

[3] Dr. R. Tourneur.

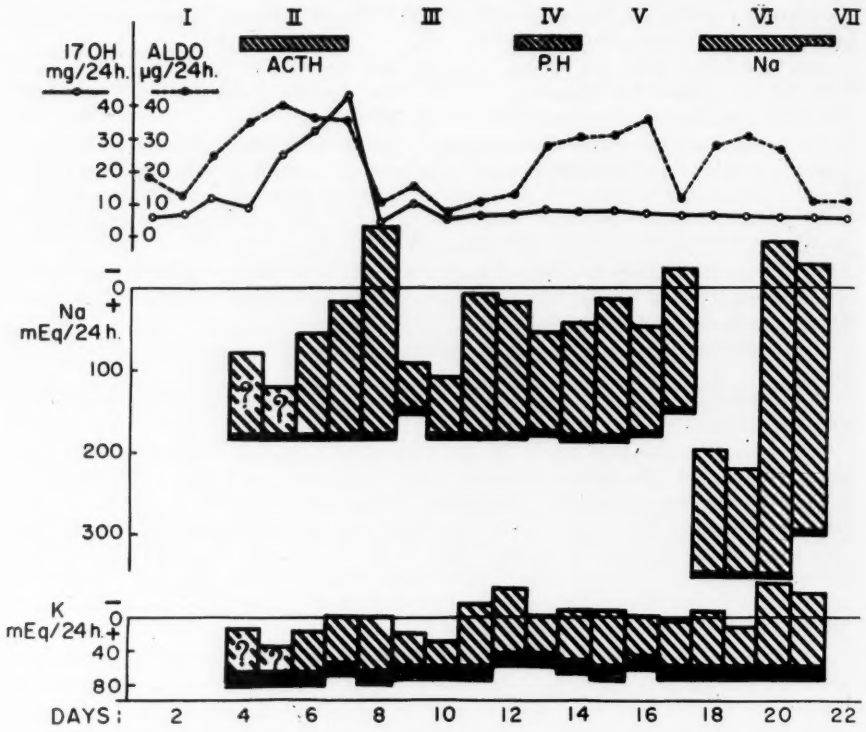

FIG. 1. Sodium and potassium balances correlated with urinary 17-hydroxysteroids and aldosterone.

potassium intake (averaging 80 mEq.). In period I intake of Na and K was very nearly that in period II, but was not definitely measured; during periods VII, VIII, IX and X preoperatively, and periods XIII and XIV postoperatively, the diet was not controlled.

In period II, purified ACTH[4] was given from the second half of day 4 to the first half of day 7 by two 8-hour intravenous infusions (50 and 30 I.U. respectively) and 4 intramuscular injections (25 I.U. each). In period IV, posterior pituitary extract[5] (34 I.U.) was given twice daily by subcutaneous injection, from the second part of day 12 until the first part of day 14. In period VI, a sodium load consisting of an oral daily intake of 170 mEq. (days 18, 19 and 20) and 120 mEq. (day 21), was given in the form of sodium chloride in addition to the basic dietary intake of 180 mEq. per day. In period VIII, 9α-fluoro-Δ^1-cortisol (9α-F-ΔF)[6] was given orally in a dosage of 2.5 mg. per day on the twenty-third and twenty-fourth days, and 5 mg. on the twenty-fifth day, in fractional doses of 0.5 mg. Cortisone acetate was given intramuscularly during the first four post-

[4] Organon-Res. P., 1159: C.O.C., gift of Mr. R. Henry and Organon (Holland).

[5] Organon Piton, 344–790, gift of Mr. R. Henry and Organon (Holland).

[6] 9α-Fluoro-1,4-pregnadiene-11β,17α,21-triol-3,20-dione, gift of the Société des Antibiotiques de France.

operative days (day 50, 125 mg.; day 51, 50 mg.; day 52, 50 mg.; and day 53, 25 mg.). Prednisone was given orally when the patient had thrombophlebitis (days 65–75).

Biochemical

Sodium and potassium in fluids and tissues were measured by flame photometry, the tissues first being treated by the Lowry-Hastings mineralization (9).

Steroid determinations were made on 24-hour urine collections preserved with chloroform and mercurithiosalicylic acid. Each 24-hour specimen was analyzed immediately after collection. Urinary creatinine was measured by the picric acid reaction of Jaffe.

A. *Group determinations*

1. On the dichloromethane neutral extract after enzymatic hydrolysis by β-glucuronidase (1000 Fishman units per ml.) and sulfatase(s) (1000 units per ml. titrated by phenolphthalein disulfate) of Helix pomatia digestive juice, the following determinations were make: 17-hydroxycorticoids (17,21-dihydroxy-20-ketosteroids) by the Silber-Porter method (10), DNPH corticoids by the 2,4-dinitrophenylhydrazine reagent of Gornall-MacDonald (11), acetaldehydogenic steroids by the Cox procedure (12), and pregnandiol by the Crépy-Meslin-Jayle technique with the Talbot sulfuric reagent (13).

2. On the phenolic fraction of an ether extract after the same enzymatic hydrolysis the phenolsteroids were measured by the Kober reaction, using the method of Jayle *et al.* (14).

3. The neutral 17-ketosteroids were measured by the Zimmermann reagent after hot acid hydrolysis (15) and ether extraction.

4. In an additional special series of determinations (Table 2), extracts of the urines were obtained by the Edwards-Kellie-Wade procedure (16). The extracts were then submitted to enzymatic hydrolysis. The foregoing group analyses were also made, and in addition: formaldehydogenic substances by the Daughaday technique modified (17), "B.T." corticoids by the blue tetrazolium reagent of Chen *et al.* (18), and hydroxysteroids ("neutral acetylable lipides") by the Weinmann-Jayle procedure (19). This last method determines the total acetylable hydroxyl groups in the neutral lipid extract and so is an index of the total steroidal excretion.

B. *Individual determinations*

1. Δ^4, *3-ketosteroids.* A 48-hour acid hydrolysis (pH 1.2) at room temperature with continuous dichloromethane extraction was employed for separation of these steroids.[7] Aldosterone was measured by the physicochemical method of Neher and Wettstein (20) slightly modified by the addition of a benzene-water partition (21). A similar method was employed for the determination of cortisol, cortisone, corticosterone and 17-hydroxycortexone, *i.e.*, double paper chromatography in appropriate systems (22, 23) and semiquantitative visual determinations (24). The identities were assumed after several control runs with appropriate standards developed by blue tetrazolium soda fluorescence (25) and phosphoric acid techniques (26), as well as the chromatography of the acetates.

2. *Hydrogenated metabolites.* After enzymatic hydrolysis and dichloromethane extrac-

[7] Aldosterone = 4-Pregnene-11β,21 diol-3,20 dione-18-ol.
Cortisol = 4-Pregnene-11β,17α,21-triol-3,20-dione.
Cortisone = 4-Pregnene-17α,21 diol-3, 11,20-trione.
Corticosterone = 4-Pregnene-11β,21 diol-3,20-dione.
17-Hydroxycortexone = 4-Pregnene-17α,21-diol-3,20-dione, or Reichstein's compound S.

tion the neutral extracts were chromatographed. Several adapted paper chromatography systems were employed to separate the metabolites of cortisol-cortisone, corticosterone, 17-hydroxycorticosterone, Reichstein's compound S and adrenal C-19 steroids (22, 23, 27, 28). The α-ketolic compounds were stained by blue tetrazolium, and pregnanetriol[8] was isolated and visualized by the trichloracetic-acid reagent (29). Controls of identity were made by several color tests and chromatographic procedures; the measurements were semiquantitative by visual comparison with standards (24, 28).

RESULTS

1. *The baseline hormonal pattern* (Tables 1, 2 and 3; Figs. 2 and 3).

a. *Hyperaldosteronism* was moderate but constant. Normal values with the method are less than 12 μg. per twenty-four hours for normal active subjects taking a normal diet. The reduced physical activity, the relatively high sodium intake, the administration of moderate amounts of potassium and the permanent hypokaliemia (less than 2.9 mEq. per liter) (5) lent additional significance to the presence of hyperaldosteronuria in our case. The excretion of aldosterone was variable (11–25 μg. per twenty-four hours).

b. *17-Hydroxycorticoids* (as measured by Porter-Silber chromogens) were in the upper range of normal. *Cortisol* and *cortisone* were in the low range of normal. The basal excretion of cortisone was greater than that of cortisol, contrary to our observations in normal women (24). The tetrahydrogenated metabolites[9] (tetrahydrocortisone, tetrahydrocortisol and allotetrahydrocortisol) were also in the low normal range. We did not find *compound S* (less than 10 μg. per day), and its main metabolite (tetrahydro S[10]) was in the normal range. This result is important from the standpoint of the mineralo-corticoid effect of compound S.

c. *Corticosterone* excretion (5 μg. per twenty-four hours) was not elevated, and its metabolites[11] (tetrahydrocorticosterone, tetrahydro A and allotetrahydrocorticosterone) were in the normal range, as also is shown by the relationship of the group determinations of the DNPH-corticoids to the 17-hydroxycorticoids. We did not find any *cortexone*[12] (less than 10 μg. per day).

d. The pattern of *17-ketosteroid* excretion was compatible with an ad-

[8] Pregnanetriol = 5β-Pregnane-3α,17α,20α-triol.

[9] Tetrahydrocortisone = 5β-Pregnane-3α,17α,21-triol-11,20-dione.
Tetrahydrocortisol = 5β-Pregnane-3α,11β,17α,21-tetrol,20-one.
Allotetrahydrocortisol = 5α-Pregnane-3α,11β,17α,21-tetrol-20-one.

[10] Tetrahydro-S = 5β-Pregnane-3α,17α,21-triol-20-one.

[11] Tetrahydrocorticosterone = 5β-Pregnane-3α,11β,21-triol-20-one.
Tetrahydro A = 5β-Pregnane-3α,21-diol-11,20-dione.
Allotetrahydrocorticosterone = 5α-Pregnane-3α,11β,21-triol-20-one.

[12] Cortexone = 4-Pregnene-21-ol-3,20-dione.

TABLE 1. URINARY STEROIDAL DATA

Period	0	I			II				
Day	**	1	2	3	4	5	6	7	8
	Base-line				ACTH 50 u i.v. 30 u i.v. 50 u i.m.				(menses)
Urine volume (ml./24 hrs.)	1,325	850	1,150	1,800	1,800	1,350	2,020	2,120	2,680
Creatinine (Gm./24 hrs.)	0.95	0.88	1.25	1.44	1.06	0.92	0.95	1.04	1.1
17-Ketosteroids (mg./24 hrs.)	0.5	6.4	6.2	10.1	5.6	8.5	15.1	23.3	16.1
17-OH-corticoids (mg./24 hrs.)	5.6	7.4	7.7	10.5	7.6	24.5	31.5	45.5	8.0
DNPH-corticoids (mg./24 hrs.)	11.8	8.8	10.8	14.4	11.2	24	31.9	44	12.3
Aldosterone (μg./24 hrs.)	25	19	11	25	36	40	36	35	10
Cortisol (μg./24 hrs.)	—†	10‡	10‡	10‡	10‡	170	180	240	11
Cortisone (μg./24 hrs.)	—	11	10	30	20	80	80	75	20
Corticosterone (μg./24 hrs.)	—	5	5	5	—	—	150	—	—

Period	III				IV		V		
Day	9	10	11	12	13	14	15	16	17
	(menses)	Recovery (menses)			Posterior Pituitary Extract 14 u	20 u	Recovery		
Urine volume (ml./24 hrs.)	2,050	1,600	2,275	2,200	1,525	1,850	1,700	1,675	2,200
Creatinine (Gm./24 hrs.)	1.0	1.07	1.23	1.02	0.92	1.08	1.07	1.12	1.04
17-Ketosteroids (mg./24 hrs.)	12.3	5.7	4.5	3.6	3.7	5.2	4.6	3.9	6.1
17-OH-corticoids (mg./24 hrs.)	9.6	6.4	6.9	6.8	8.2	7.5	7.6	6.7	6.8
DNPH-corticoids (mg./24 hrs.)	11.3	9.4	10.2	9.9	11.8	9.4	10.6	11.0	10.6
Aldosterone (μg./24 hrs.)	15	7	11.5	13	28	30	31	36	12
Cortisol (μg./24 hrs.)	2	4.5	6.5	4.5	7.5	5	6.5	8.5	5.0
Cortisone (μg./24 hrs.)	29	10	14	13	20	28	28	36	18
Corticosterone (μg./24 hrs.)	—	3	3	3	—	3	3	—	—

Period	VI		VII			VIII		
Day	18	19	20	21	22	23	24	25
	Sodium load 350 mEq.	350 mEq.	350 mEq.	Sodium load 300 mEq.		2.5 mg.	9αF—Δ¹F 2.5 mg.	5 mg.
Urine volume (ml./24 hrs.)	1,880	1,550	2,940	2,320	1,380	2,220	1,475	1,880
Creatinine (Gm./24 hrs.)	0.90	0.95	1.06	0.93	1.04	0.98	1.08	0.95
17-Ketosteroids (mg./24 hrs.)	6.9	5.7	5.0	3.3	5.0	4.0	1.8	1.4
17-OH-corticoids (mg./24 hrs.)	6.6	6.7	6.0	6.2	6.2	5.2	1.0	2.7
DNPH-corticoids (mg./24 hrs.)	9.6	7.4	9.1	9.5	9.8	10.0	6.8	6.8
Aldosterone (μg./24 hrs.)	28	30	27	10	10	—	—	1.4
Cortisol (μg./24 hrs.)	10	—	13	3.5	4	—	—	1
Cortisone (μg./24 hrs.)	15	21	30	15	9	—	—	0.8
Corticosterone (μg./24 hrs.)	—	—		5	5			

Period	IX			X						
Day	26	27	28	29	30	31	32	33	34	35
			tetany	3 Gm.	5 Gm.	Potassium chloride plus free diet 5 Gm.	5 Gm.	5 Gm.	5 Gm.	3 Gm.
Urine volume (ml./24 hrs.)	3,025	835	990	960	1,220	2,100	2,200	2,700	—	3,025
Creatinine (Gm./24 hrs.)	1.7	0.70	0.71	1.17	0.89	1.4	1.23	1.08	—	1.0
17-Ketosteroids (mg./24 hrs.)	2.8	3.8	4.0	5.4	4.0	6.5	4.8	3.1	—	3.7
17-OH-corticoids (mg./24 hrs.)	5.7	6.1	11.6	11.4	9.1	11.4	8.8	9.0	—	9.11
DNPH-corticoids (mg./24 hrs.)	15.1	5.7	12.6	11.7	10.6	12.0	9.9	13.5		11.2
Aldosterone (μg./24 hrs.)	1.6	7.0	—	—	—	20	—	—	—	—
Cortisol (μg./24 hrs.)	2	10.5	—	—	—	13	—	8	—	—
Cortisone (μg./24 hrs.)	4	12	—	—	—	23.5	—	10	—	—
Corticosterone (μg./24 hrs.)	—	—	—	—	—	—	—	—	—	—

** Four months before study: free diet in addition. DHEA, 0.5 mg./24 hrs.; acetaldehydogenic substances, 2.3 mg./24 hrs.; pregnandiol-complex,3.2 mg./24 hrs.; (luteal phase); phenolsteroids, 15 μg./24 hrs.

† Not measured.

‡ On a 4-day pool.

TABLE 2. INDIVIDUAL DETERMINATIONS OF HYDROGENATED METABOLITES

(see text for methods and nomenclature)

	Day 40	Day 41
Urine volume (ml./24 hrs.)	1,620	1,700
Creatinine (Gm./24 hrs.)	0.92	0.95
17-Ketosteroids (mg./24 hrs.)	1.9	4.2
17-OH-corticoids (mg./24 hrs.)	5.7	7.1
DNPH-corticoids (mg./24 hrs.)	7.9	7.5
Tetrahydrocortisol (μg./24 hrs.)	200	270
Allotetrahydrocortisol (μg./24 hrs.)	65	35
Tetrahydrocortisone (μg./24 hrs.)	400	450
Tetrahydro S (μg./24 hrs.)	20	85
Tetrahydrocorticosterone (μg./24 hrs.)	40	100
Allotetrahydro-corticosterone (μg./24 hrs.)	60	85
Tetrahydro A (μg./24 hrs.)	40	?*
Androsterone (μg./24 hrs.)**	50	70
Etiocholanolone (μg./24 hrs.)†	250	270
DHEA (μg./24 hrs.)‡	250	?*
11-Keto-etiocholanolone (μg./24 hrs.)	160	100
11β-OH-etiocholanolone (μg./24 hrs.)	320	340
11β-OH-androsterone (μg./24 hrs.)	380	370

* Lost.

** Androsterone = 5α-Androstane-3α-ol-17-one.

† Etiocholanolone = 5β-Androstane-3α-ol-17-one.

‡ DHEA = 5-Androstene-3β-ol-17-one.

renal abnormality. The total value was at the inferior limit of normal and the individual values indicated abnormal relationships among the individual 17-ketosteroids.

e. The amount of *hydroxyl groups of the neutral steroids* lay in the normal range (19) (Table 3). If we calculate the theoretical hydroxyl-microequivalents (μEq.) of the measured chemically well-defined compounds (formaldehydogenic, acetaldehydogenic and 17-ketosteroids), there is no discrepancy between the calculated and the measured values. This is assurance that no unknown hydroxysteroid was present in the neutral extract in a quantity within the limits of accuracy of the method (an order of 3 mg. per day if we assume a mean molecular weight of 300 for the steroids). A very important discrepancy, however, was the relatively high level of formaldehydogenic substances which contrasted with the levels of Porter-Silber and blue tetrazolium chromogens (low-normal). As approximately the same value for the formaldehydogenic α-ketols (20-keto-21-ol) and

TABLE 3. ANALYSIS OF 3 URINARY POOLS

(extracted by ether 3-ethanol 1 procedure)

Urinary samples→	Period I (pool of days 1, 2, 3, 4)		Period II (pool of days 5, 6, 7)		Period IV (pool of days 13, 14, 15)	
Treatment→	None		ACTH		Post. Pituitary Extract	
Excretion→	mg./24 hrs.	μEq.* /24 hrs.	mg./24 hrs.	μEq.* /24 hrs.	mg./24 hrs.	μEq.* /24 hrs.
Hydroxysteroids, measured		147		310		99
Formaldehydogenic substances (I)**	14.0	93	37.8	252	6.4	42
Acetaldehydogenic substances (II)†	4.8	28	6.2	36	2.9	17
17-Ketosteroids (III)‡	6.5	22	12.6	42	4.8	16
Hydroxysteroids, calculated from (I)+(II)+(III)		143		330		75
B.T. chromogens	2.8		10.0		1.5	
17-OH-corticoids (Porter-Silber chromogens)	2.8		8.1		1.3	

* 1 μEq.-OH indicates 1 μEq. of acetylable hydroxyl groups in the extract. In our calculation, 1 μM. of 17-ketosteroids is represented by 1 μEq.-OH, and each μM. of acetaldehydogenic steroid by 2 μEq.-OH; for the formaldehydogenic steroids we calculate 2.5 μEq.-OH/μM. of steroid in view of the existence of 20-ketonic compounds (like tetrahydro-derivatives) with values of 2 μEq.-OH/μM., and of 20-alcoholic compounds (like cortol and analogues) with values of 3 μEq.-OH/μM.

** Standard: cortisone.

† Standard: pregnanetriol.

‡ Standard: DHEA.

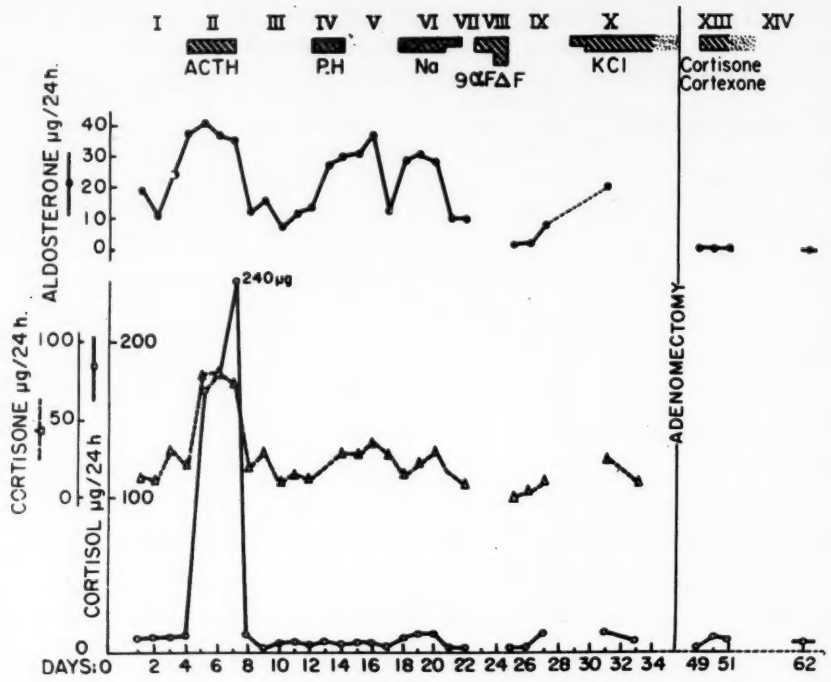

FIG. 2. Urinary aldosterone, cortisol and cortisone.

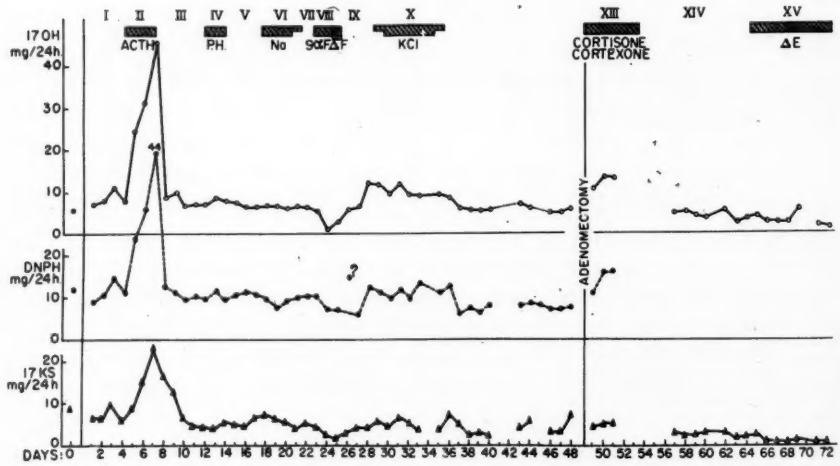

FIG. 3. Urinary 17-ketosteroids, 17-hydroxysteroids and DNPH-corticoid's.

glycols (20-21-diol)[15] is usually found, we may postulate an increase of the glycols. We can assume that either they are metabolites of an unknown substance(s) or that they are the consequence of an excessive reducing process which provokes more 20-ol than 20-keto compounds. This pattern is also found in periods during administration of ACTH and posterior pituitary extract.

f. In a recent work, Pasqualini (30) in our laboratory isolated from the preoperative urine of this patient an *unknown compound* (Δ^4, 3-ketosteroid ?) very similar to the compound III of Nowaczynski *et al.* (31) which was found also by Crabbé *et al.* (32). The major percentage, if not all of the compound, is eliminated in a conjugated form which is not hydrolyzed by the pH1 method used for our aldosterone determination. Hence, it does not interfere with our aldosterone values. Details on this work will appear later (33).

2. *The control(s) of hormonal secretions*

a. *Effects of ACTH.* The levels of aldosterone excretion on days 4, 5, 6 and 7 (mean, 36 μg. per twenty-four hours) and the subsequent decrease after cessation of stimulation indicate augmentation of aldosterone production, most probably from the tumor; however, a contribution from the normal adrenal tissue cannot be rigorously excluded. At the same time, the effect of ACTH on cortisol, cortisone, 17-hydroxycorticoids, DNPH-corticoids and 17-ketosteroids was quantitatively and qualitatively normal; the percentage increase was definitely much higher for all these adrenal metabolites than for aldosterone. The increase of aldosterone showed a sudden rise to a maximum and fell concomitantly with the return of the different metabolites of cortisol to normal.[16] The precocity of the increase of aldosterone, however, was in correlation with the positive sodium balance at the beginning of ACTH administration (Fig. 1). When the increase of glucocorticoids progressed, the sodium balance was normalized and became negative only when aldosterone decreased. The question of ACTH control of aldosterone secretion has been recently reviewed (34, 35). Müller and Mach (36) claim that stimulation of aldosterone by ACTH is

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[15] Formaldehydogenic steroids (F) are 20,21-glycolic and 20,21-ketolic steroids. The α-ketolic steroids are measured by the blue tetrazolium reaction (B). Hence we may assume that the difference (F)–(B) represents the 20,21-glycolic steroids. This assertion is supported by the fact that when normal urine extracts are divided into ketonic and nonketonic fractions, the B.T. chromogens are found only in the ketonic fraction, and the formaldehydogenic steroids are found equally in each fraction.

[16] J. Genest (Montreal) has recently observed the same phenomenon (personal communication.

immediate, and afterwards it is antagonized by retention of sodium and water and/or a concomitant increase in 17-hydroxycorticoids. In our patient their explanation seems compatible with the observed facts.

b. *Effects of posterior pituitary extract* (*Piton*). The administration of Piton was followed by an increase in aldosterone excretion which was not immediately maximal but seemed to persist after administration ceased. Here also the initial rise of aldosterone was correlated with a positive sodium balance (Fig. 1).

There was no other change in steroids during the administration of the posterior hypophyseal extract. These results, to our knowledge, are the first obtained in man which corroborate the *in vitro* findings of Giroud *et al.* (37); the direct effect of a posterior-hypophyseal aldosterone-stimulating factor can be observed here because no conflicting water retention occurred in this study as would ordinarily occur in normal subjects. In normal subjects aldosteronuria decreases "secondarily" by increasing extracellular fluid volume (38).

c. *Effects of a sodium load.* Assuming that the isolated pretreatment value (12 μg.) reflects the return of urinary aldosterone to the basal level, the administration of a sodium load invoked an initial paradoxical increase followed by a decrease in aldosterone excretion on the third day, in contrast to the constancy of elimination of the 17-hydroxycorticoids, DNPH-corticoids and 17-ketosteroids. However, the reduction in cortisol-cortisone excretion was an unexpected and unexplained finding. The decrease in aldosterone output was delayed by comparison with that in normal subjects (36) and the depressed level was still abnormally high. Hyperaldosteronism was sensitive to this metabolic modification, but an abnormally high level of secretion was maintained throughout.

d. *Effects of 9α-fluoro-Δ¹-cortisol* (*9αF-Δ¹F*). This synthetic compound is very effective in suppressing adrenal secretion. The expected results were obtained in regard to the elimination of 17-hydroxycorticoids, cortisol, cortisone, DNPH-corticoids and 17-ketosteroids; they were probably suppressed via inhibition of ACTH secretion. This observation confirms the normal biogenesis and regulation of glucocorticoids and androgens in primary hyperaldosteronism, as was noted during the baseline and the ACTH-stimulating periods.

An extraordinary effect of 9αF-Δ¹F was the marked decrease in aldosterone excretion (less than 2 μg. per twenty-four hours), with a return to the previous level after cessation of administration. Although the recovery period following the sodium load test was unfortunately short, the validity of aldosterone suppression is supported by the return to the basal level of

aldosterone immediately after discontinuance of 9αF-Δ^1F, and the fact that the reduction in sodium intake on day 21 did not exert any influence on aldosterone excretion, which was constant on days 21 and 22. On day 28 a severe tetanic crisis occurred, aldosterone excretion having returned to the basal level at that time. Three possibilities exist to explain these variations of aldosteronuria:

1) A direct effect of 9αF-Δ^1F on the biogenesis of aldosterone in the tumor. This is improbable, considering what is known about the mechanism of action of this class of "cortisonoid compounds."

2) An indirect inhibition via ACTH stimulation. It is striking that the aldosterone production of such an adenoma should be under the control of ACTH, as are the glucocorticoids and androgens. In any case, the conflict of this hypothesis with the findings of some animal and human experiments is obvious (review, (34)).

3) Mediation through a metabolic effect of the 9α-fluoro-Δ^1-cortisol is possible, especially via a gross depletion of potassium. The tetanic crisis at the time of the return to a baseline aldosterone level would argue for this premise. However, the concentration of serum potassium was not changed on that day; unfortunately, no balance study of electrolytes was made during this period. The effect might have also been mediated through an expansion of the extracellular fluid, in view of a post-treatment diuresis.

Comment. Whatever the mechanism(s), the aldosterone secretion of this adenoma was probably not autonomous, since participation of the nontumorous adrenal remnant to produce the foregoing changes seems unlikely.

3. *Metabolic data correlated with steroidal studies (Fig. 1) (Table 1)*

Baseline state (Period I). The total extracellular sodium as calculated from the sodium concentration in the plasma and the extracellular space (13.9 L. or 26 per cent of body weight by mannitol diffusion) was found to be in the upper range of normal. The intracellular sodium measured in the skin and abdominal muscles appeared slightly elevated; however, when subjected to statistical analysis, these values were found not to be significantly elevated (Table 4). Also, the finding that the Na^{24} diffusion space (13.1 L. or 24.3 per cent of body weight) was equal to the mannitol diffusion space argued against a grossly abnormal rate of entry of sodium into the intracellular space (total water by antipyrine diffusion, 33.4 L. or 62 per cent of body weight).

As is usually reported in this syndrome, the level of sodium was approximately normal in the urine and elevated in the saliva. Hence, the total

TABLE 4. SODIUM AND POTASSIUM IN TISSUES*

Fresh tissue		Potassium (mEq./Kg.)				Sodium (mEq./Kg.)			
		Mean	No. of samples	S.E. of mean	*t* test	Mean	No. of samples	S.E. of mean	*t* test
Skin	36 normal subjects	19.5	36	0.78	2.60	91	36	2.52	1.28
	Mrs. G., at operation	12.8	3			105.3	3		
	Mrs. G., 9 day postop. (day 58)	22	2			96	2		
Muscle	4 normal subjects	83.1	9	0.80	4.95	37.3	9	1.85	2.09
	Mrs. G., at operation	73	2			46.1	2		

* A. Reinberg.

body sodium was increased only slightly (if at all), and the normal physiologic ratio between the extra- and intracellular compartments was evidently not disturbed.

Potassium deficiency was, on the contrary, quite pronounced. The hypokaliemia was profound, and in the tissues the deficit was highly significant statistically (Table 4). An elevated renal clearance for potassium appeared to be the mechanism by which this deficiency was maintained in the presence of an equilibrated potassium balance. Salivary potassium concentration was high and the fecal output was normal[17] as in Conn's (1) and Mader-Iseri's cases (7) and contrary to the findings in the "mixed case" of Brooks *et al.* (39).

The over-all status of sodium and potassium was a sustained loss of potassium from the cells and plasma by way of the urine and saliva, in contrast to a questionable retention of sodium in the intracellular and extracellular compartments. Thus the increase in tissue sodium may be only compensatory to potassium loss, and urinary sodium excretion may arrest further sodium retention. This was the picture along with the isolated abnormal steroid finding of moderately elevated excretion of aldosterone (11–25 μg. per twenty-four hours).

ACTH (Period II). Initially, ACTH caused sodium retention and an elevation of urinary aldosterone; the 17-hydroxycorticoids increased one day later (day 5). This supports Conn's assertion about the role of aldosterone in the initial ACTH-induced sodium retention (1). However, at this time an unexpected finding was the positive potassium balance. In the second phase (days 6–7) aldosterone and corticoids were elevated, and sodium diuresis commenced. Potassium balance at this point tended to reach a state of equilibrium. The increase in water, sodium, potassium and

[17] Measured only on days 20, 21 and 22 (13 mEq., 12 mEq. and 9.5 mEq.).

chloride diuresis was similar to the Mader-Iseri findings with cortisone (7). On day 8, the sodium diuresis was maximal, but aldosterone and 17-hydroxycorticoids (cortisol metabolites) had already returned to their initial values. Thus, these data do not definitely support Conn's view on cortisol-aldosterone antagonism as a factor in the sodium diuresis (1); the simultaneous variations in both these steroids do not permit such a conclusion on this point.

Immediately after the natriuresis, there was a marked retention of sodium (days 9–10), as previously shown by Conn (1). (It should be noted that during this period aldosterone excretion had returned to a basal level, as had the excretion of other corticoids; also, the potassium balance was positive.) Then, without any steroidal modification, the basal sodium-potassium balance was restored in the following two days (days 11 and 12). The water balance closely paralleled the electrolyte balance, and the plasma sodium level temporarily increased during this period. No explanation yet proposed had been successful in clarifying the data of Period III.

Posterior pituitary extract (Piton) (Period IV). Piton seemed to induce simultaneously a retention of sodium and an increase in aldosterone secretion, while 17-hydroxysteroid excretion remained constant. This sodium retention is the opposite of the effect of Pitressin in normal subjects (38). In the latter, water retention leads to a decrease in aldosterone excretion, but in our patient the relative insensitivity of the tubular cells to Pitressin led to a direct demonstration of Pitressin-induced hyperaldosteronism. During this period, potassium balance was approximately stable. On day 17, aldosterone excretion decreased and the sodium balance was negative.

Sodium load (Period VI). The major effect was a repid excretion of the load, identical to that in a normal subject. Initially there was retention of sodium accompanied by a paradoxical increase of urinary aldosterone (28 μg. per day) as in the case of Duncan *et al.* (40). Corticoids were unchanged. The sodium diuresis began *before* the decrease in aldosterone. Moreover, after an initial increase in the loss of potassium (day 18), the potassium balance paralleled that of sodium. On days 18 and 19, when sodium and water retention occurred, plasma concentrations of potassium and sodium were slightly depressed. Thus aldosterone excretion was sensitive to variations in sodium, as it is in normal men (19, 38, 40–42).

Comment. In these dynamic balance studies, even if the sodium load were not considered (days 18 and 19), no correlation could be established between urinary sodium or Na/K ratio and urinary aldosterone. This is contrary to the findings in normal (5, 43) and in various pathologic (44) subjects.

With the foregoing exceptions, there was a general parallelism of the sodium and potassium balances as also seen by Brooks *et al.* (39). This

parallelism seems contradictory to a thesis of an exclusive aldosterone effect, which should provoke an opposite effect on the potassium and sodium balances (4, 45).

DISCUSSION

1. In regard to the first question raised, "What is the total steroidal pattern of this syndrome?," a partial answer is now evident.

A re-assessment (Table 5) of cases showing potassium loss, arterial hypertension, hyperaldosteronuria, and adrenal anatomic abnormality is useful for a more exact classification of our findings. We suggest that the name "primary hyperaldosteronism" be applied to cases in which urinary 17-ketosteroids and 17-hydroxysteroids are normal. Conn did not measure corticosterone or its metabolites in his inaugural case (1), but other investigators in similar cases found corticosterone to be either normal (3, 4, 6) or elevated (3, 4, 6, 7, 48). Therefore, when corticosterone levels are normal we suggest the term "pure primary hyperaldosteronism," and when they are elevated, the term "primary hyperaldosteronism with hypercorticosteronism" In some other cases, no corticosterone determinations were made, and we are not able to classify them (1, 49, 50, 55). Three cases (39, 56, 57) have been described in which 17-ketosteroids, 17-hydroxysteroids, 17-ketogenic steroids or other steroids were high; for these, we propose the name of "mixed hypercorticism including hyperaldosteronism" (Table 5).

In this sense our present case would be "pure primary hyperaldosteronism." We found no abnormalities of the classical "glucocorticoids" (cortisol, cortisone, corticosterone) and so-called "mineralocorticoids" (corticosterone, 17-hydroxycortexone and cortexone). The regulation by ACTH (both stimulation and inhibition) of corticoids and androgens appeared to be normal. Also, the metabolites of progesterone and 17-hydroxyprogesterone were within normal limits. Some minor modifications of the 17-ketosteroid pattern were probably not specially significant, as they are common in other adrenal disturbances very different from hyperaldosteronism. Determinations of hydroxysteroids indicated an excess of unknown formaldehydogenic compounds, probably 20,21-glycols; we do not know the significance of this abnormality.

2. In answer to our second question, "Is the adenoma autonomous, or is its secretion(s) subject to control(s) by some hormonal or humoral changes in the body?" we can state definitely that its secretion was influenced by several hormonal and humoral factors. The data strongly suggest the influence of ACTH. The effect of 9α-fluoro-Δ^1-cortisol was definite and might have been mediated by a hormonal (ACTH decrease) and/or a humoral (potassium depletion) factor(s).

TABLE 5. TENTATIVE CLASSIFICATION OF PUBLISHED CASES OF "PRIMARY ALDOSTERONISM"*

(with a summary of the data from the literature on steroidal studies, aldosterone not included)

	Sex of patient	Adrenal pathology	Urinary 17-KS (mg./day)†	Tumoral or urinary cortisol (or derivatives)**	Tumoral or urinary corticosterone (or derivatives)***	Other results†	Author, Case No., & Reference
Pure primary hyperaldosteronism	F	adenoma	—	1b→†	2b→†	no cortexone in urine; plasma-cortisol→	Ayres *et al.* 1‡ (4)
	—	adenoma	—	1b→	2b→	——	Ayres *et al.* 2 (4)
	—	adenoma	—	1a→	2a→	no excess "S"	Neher 2/2 (6)
	—	adenoma	—	1a→	2a→	no excess "S"	Neher 2/3 (6)
	F	adenoma	8.5	1b→ 1d→	2b→ 2c→	see text	Present case
Primary hyperaldosteronism with increase of corticosterone	F	adenoma	8.8	1d↗+	2a↗++ 2c↗++	Pettenkofer chromogen→	Mader & Iseri (7)
	F	adenoma	6.6–7.8	1e→	2c↗++	ACTH response→	Skanse *et al.* (48)
	—	adenoma	—	1a→	2a↗++	——	Ayres *et al.* (4)
	—	"tumor"	—	1a→	2a↗++	no excess "S"	Neher 2/1 (6)
Primary hyperaldosteronism; no measurement of corticosterone	F	adenoma	5.7	1d→	?	ACTH response→	Conn (1)
	M	adenoma	12	1f→	?	——	Hewlett *et al.* 1 (49)
	F	adenoma	10.6	1f→	?	——	Hewlett *et al.* 2 (49)
	F	adenoma	7.5	1d→	?	——	Mucio *et al.* (50)
	M	adenoma	2–8	1d→	?	——	Hoet (51)
	F	adenoma	4.3	1d→	?	plasma cortisol→	Eales & Linder (52)
	M	adenoma	8.2.–12.0	1d→	?	——	Fine *et al.* (53)
	M	adenoma	→	?	?	——	Bruhl (54)
	M	hyperplasia	11.5	1d→	?	pregnandiol, 2.7 mg./day chromatography 17-KS→ acid-ketonic steroids→	van Buchem *et al.* (55)
Mixed hypercorticism, including hyperaldosteronism	M	cancer	29	1e↗++	?	——	Brooks *et al.* (39)
	M	cancer	6–15 278§	1e↗++ 1e↗++	? ?	——	Foye & Feichtmeir (56)
	M	hyperplasia	11	1d↗++	?	11-oxygenated steroids↗++	de Moor *et al.* (57)

* The cases of Crane *et al.* (66) (63) and Goldsmith *et al.* (67) (64) differ by the presence of edema and are not included in this table (see text, discussion *3*).

† The results are classified by the range of normal of the particular laboratory in which each investigation was carried out:

→ normal value;
↗+ slightly increased value;
↗++ increased value.

** 1a = cortisol in tumor.
1b = cortisol in urine.
1c = free 17-hydroxycorticosteroids (Porter-Silber chromogens) in urine.
1d = total 17-hydroxycorticoids (Porter-Silber chromogens) in urine.
1e = 17-ketogenic corticoids which, in these cases, may be considered as an index of cortisol metabolites.
1f = unknown technic.

*** 2a = corticosterone in tumor.
2b = corticosterone in urine.
2c = blue tetrazolium corticoids which, in these cases, may be considered as an index of corticosterone metabolites.

‡ Ayres' Case 1 = Chalmer's patient (47).

§ During recurrence of tumor after surgery.

The effect on aldosterone of a factor contained in posterior pituitary extract has an important biologic significance. In the rat we recently demonstrated with Mialhe-Voloss (58, 59) the effect of "posterior hypophyseal ACTH" and of corticotropin-releasing factor ("CRF") on aldosterone production, but in this animal these substances (directly for the former and via ACTH release for the latter) had the same effects as did "anterior hypophyseal ACTH" and also increased corticosterone secretion. In our case, on the contrary, the posterior pituitary extract acted only on aldosterone and not on other corticosteroids. Perhaps the finding of Venning *et al.* (60) that emotional disturbances increase the excretion of aldosterone but not cortisol is also in agreement with the neuro-posterior pituitary regulation of aldosterone secretion.

The non-autonomous biogenesis of steroids in the adenoma is an argument in favor of the development of the tumor by way of a non-tumoral hyperplastic state, as we have suggested for Cushing's syndrome (61) on the basis of substantiating biochemical and anatomic data.

3. As for the third question, concerning metabolic-steroidal correlations, a definite conclusion cannot be reached from existing data.

Obviously the physiologic properties of aldosterone do not fully explain the abnormalities of sodium-potassium metabolism in "pure primary hyperaldosteronism." August, Nelson and Thorn (62) have proposed an "escape" of the aldosterone effect to explain the lack of edema in the syndrome described by Conn. However, in states of hypokaliemia brought about by massive electrolyte losses, similar Pitressin-resistant polyuria and anatomic renal alterations are found together with a tendency towards sodium retention and edema without hyperaldosteronuria. An alternative explanation is that, in addition to hyperaldosteronism, there is an excess of a "sodium-excreting factor." Analysis of the urine of our patient by Dr. R. Neher[18] for 5α-pregnane-3β,16α-diol-20-one, a sodium-excreting factor recently isolated (63), showed a value less than in normal urine. However, other sodium-excreting factors have been described (64), but they were not measured in this patient. In 2 recent cases of "primary aldosteronism," the occurrence of edema was noted by Crane (65), Goldsmith (66) and their associates. In the latter instance cortisone and cortexone were measured in the tumor tissue and found to be elevated. Moreover, it is well known that certain adrenal syndromes cause hypokaliemia without hyperaldosteronuria (67, 68).

Further studies are necessary to correlate metabolic and steroidal findings. We have endeavored to draw attention to uninterpretable data in a syndrome that at first appeared simple.

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TWO UNILATERALLY CRYPTORCHID BOYS WITH SPERMATOGENIC PRECOCITY IN THE DESCENDED TESTIS, HYPERTELORISM AND POLYDACTYLY

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ABSTRACT

Two boys, aged 4 and 6 years, had the following features in common: unilateral cryptorchidism, round bodies in the seminiferous tubules of the undescended testis, primary spermatocytes in the scrotal testis, hypertelorism, and reduplication of one digit on the same side as the cryptorchidism.

TWO boys with multiple congenital abnormalities have been observed in whom the gonadal factors seemed particularly noteworthy. In each case the undescended testis showed gonadal dysgenesis, whereas the descended testis showed premature maturation of the germinal epithelium. Hypertelorism and polydactyly were also present.

CASE HISTORIES

Case 1

D. H., aged 4 years, entered the clinic with a right inguinal hernia which had been present since birth. A normal delivery had followed an uncomplicated full-term pregnancy and the developmental pattern of the child had been considered normal. Past medical history revealed that several cousins had extra digits.

Physical examination showed a well nourished, cooperative white child whose height was 1.0 M. and weight 16.5 Kg. The head was rather large and measured 55 cm. in circumference. Patchy areas of vitiliginous and ichthyoid skin were noted on the right side of the body from the chest to the foot. The eyes were widely separated and there was right strabismus. The heart presented no abnormalities except a grade II soft systolic murmur. The right testis could not be palpated in the inguinal or scrotal areas. The left testis was in the scrotum and measured $1.5 \times 1.0 \times 1.0$ cm. A partial supernumerary right great toe was noted (Fig. 1).

Laboratory examination: Results of routine tests were within normal limits. The low incidence of sex chromatin in the cells of the buccal mucosa was within the male range. Urinary 17-ketosteroids in two 24-hour collections of urine were within normal limits (3.4 mg. and 3.3 mg. per Gm. of urinary creatinine). The gonadotropin extract of the pooled 48-hour specimen of urine was injected into an immature female rat, resulting in an ovarian weight of 66 mg. The interstitial cells of the sectioned rat ovaries were hypertrophied. The urinary gonadotropin was therefore believed to be interstitial-cell stimulating hormone (ICSH).

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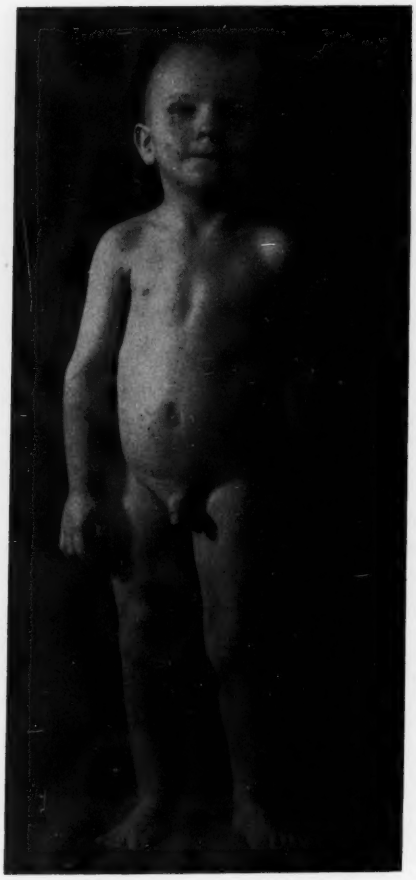

FIG. 1. Patient D.H. (Case 1), aged 4 years; height 1.0 M., weight 16.5 Kg. Note large head, wide separation of the eyes, right strabismus, and partial reduplication of the right great toe.

X-ray films of the skull demonstrated "increased size of the calvarium with some asymmetry." There was a moderate degree of undercutting of the anterior clinoids; the sella was of normal size. The greater wings of the sphenoid were large and hypertelorism was present. Failure of fusion of the posterior arch of C1 was noted. The impression was "hydrocephalus without evidence of increased intracranial pressure." X-ray examination of the hand and wrist demonstrated that osseous carpal development corresponded to that found at the age of 3 years, indicating retardation.

A testis with its epididymis and vas deferens was found in the right inguinal canal. Biopsy and orchidopexy were performed. Tissue also was removed from the left descended testis.

Microscopic examination of the biopsy specimen from the right testis showed gonadal dysgenesis, *i.e.*, undifferentiated seminal cords and intratubular bodies (Fig. 2). Histologic sections of tissue from the left testis showed tubules with primary spermatocytes (Fig. 3); this stage of maturation is not usually seen until the age of 12–14 years.

When the patient was re-examined three months postoperatively, the right testis was freely movable in the scrotum. Neurologic investigation failed to demonstrate any intracranial lesion. The 24-hour excretion of 17-ketosteroids was normal (4.8 mg.). In the gonadotropin assay, injection of the total extract of urine into the immature female rat produced ovaries weighing 24 mg. Ovarian sections showed stimulation of the thecal cells.

Case 2

B. P. (case of Dr. E. J. Marble) was 6 years old. His weight at birth was 5 pounds 1 ounce. Because of delayed closure of the anterior fontanelle, he had been followed in the outpatient service of another hospital. X-ray films of the skull taken at the age of 2 years were interpreted as showing: "No abnormalities involving the skull. The sutures are normal. The anterior fontanelle is still widely open. The sella is normal. There is no intracranial calcification." X-ray films of the hand indicated "a bone age of 24 months." At the age of 4 years the anterior fontanelle tightly admitted one finger. The weight was 27½ pounds, and the height 36 inches, resulting in a weight-age of 1½ years and a height-

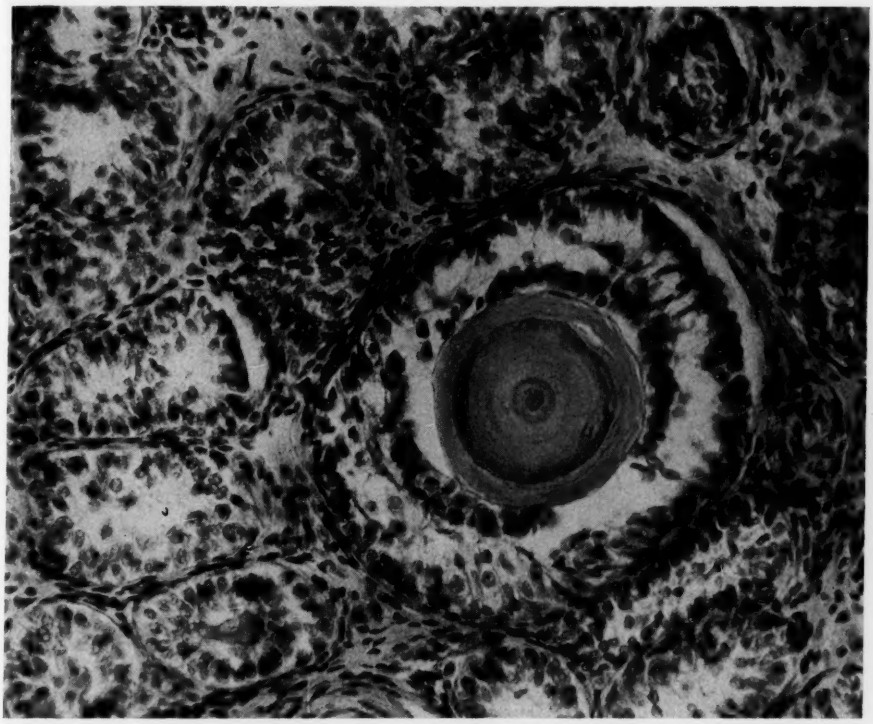

FIG. 2. Patient D. H. (Case 1). Histologic section of tissue from right (undescended) testis. The tubules are small (60–80 microns in diameter) and contain small cells. Occasional tubules contain a few spermatogonia. A much larger tubular cross-section contains a round body with a core. The core is surrounded by concentric rings of materials varying in density. The outermost ring stains lightly with eosin. The sustentacular cells are numerous and arranged in two layers; an occasional germinal cell is present among them. The interstitium is undifferentiated. In other sections an occasional seminal cord could be seen.

age of $2\frac{1}{2}$ years. He was considered a "very alert lad of normal intelligence." "No symptoms referable to the central nervous system had occurred." Reduplication of the right thumb had been corrected surgically. There was no family history of congenital anomalies.

Physical examination showed a well developed and well nourished white male. The eyes were widely separated. The left testis was in the scrotum, the right was in the inguinal region. Results of routine laboratory studies were within normal limits. The right testis was described as small. Biopsy and orchidopexy were performed. A biopsy specimen was also obtained from the left, larger descended testis.

Histologic sections of tissue from the right (undescended) testis showed gonadal dysgenesis (Fig. 4), as in Case 1. Sections from the left testis showed premature maturation of the germinal epithelium (Fig. 5). A survey of the nuclear sex chromatin pattern indicated a male type. Subsequently two 24-hour urine samples yielded normal values

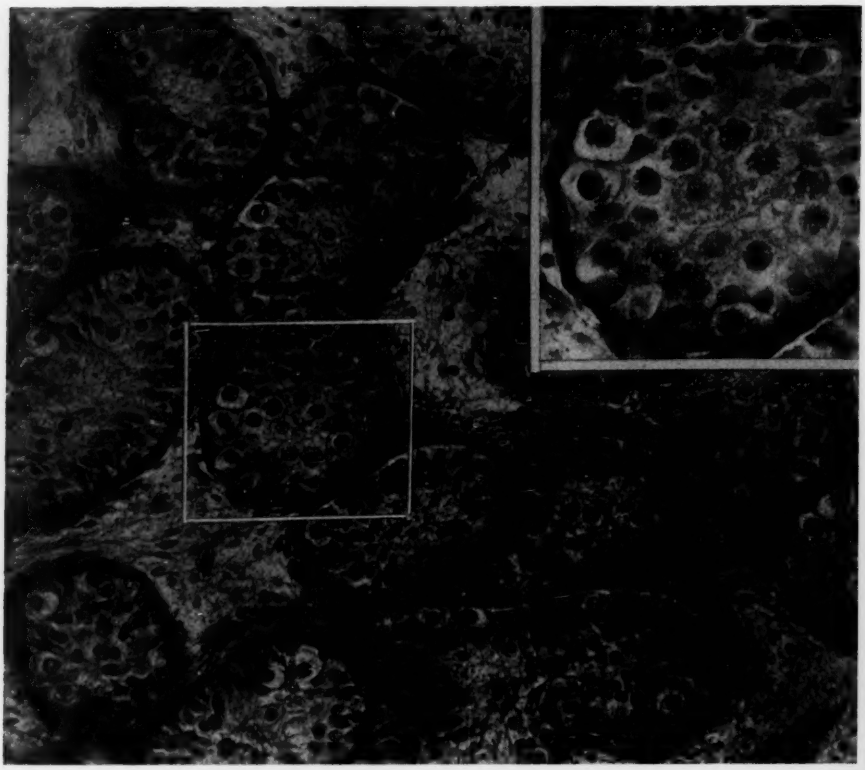

FIG. 3. Patient D. H. (Case 1). Histologic section of tissue from left (descended) testis, showing a loose undifferentiated interstitium. Tubules (80–120 microns in diameter) are larger than those of the right testis, are more tortuous, contain lumina, and have a good complement of sustentacular cells and spermatogonia. Some tubules contain, in addition, cells with thready chromatin material typical of primary spermatocytes (see insert).

for 17-ketosteroids (3.0 and 4.0 mg. per Gm. of urinary creatinine). Neither urine specimen contained demonstrable gonadotropic activity.

DISCUSSION

Patients having hypertelorism and extra digits have been previously reported. We are not aware of any case reports in which both reduplicated digits and undescended testes were observed. One patient in whom hypertelorism and gonadal dysgenesis were co-existent was reported by us (1). It may be significant that the supernumerary digit and the undescended testis were on the same side of the body in the 2 patients reported here. Dr. Ponseti of our Orthopedics Department has observed 6 patients with unilateral congenital hip deformities in whom the testis was undescended

on the ipsilateral side. Unilateral occurrence of anomalies may have some developmental significance, since our Patient D. H. (Case 1) also had vitiliginous skin on the right side of the body, and right strabismus.

Evidence of testicular dysgenesis (seminal cords, intratubular bodies (2)) in the undescended testes of these 2 patients supports the contention of those who believe that defective testes are less likely to descend, and that gonadal ectopia is not the primary etiologic factor reducing the fertility potential.

The descended testes reported here were remarkable because of the presence of primary spermatocytes which normally appear at 12 to 14 years

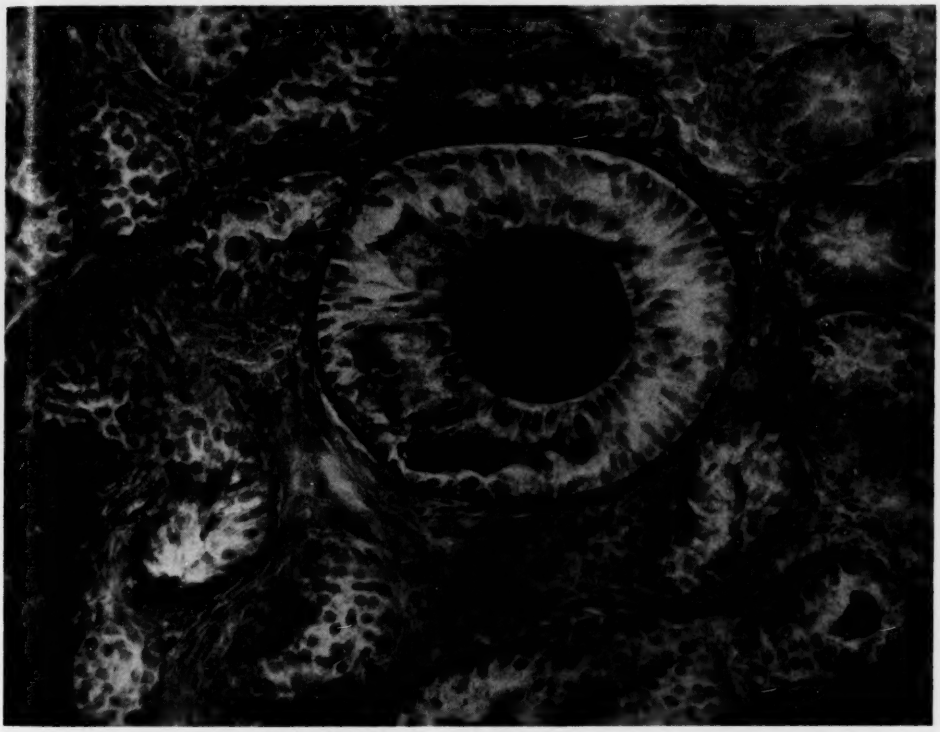

FIG. 4. Patient B. P. (Case 2). Histologic section of tissue from right (undescended) testis, showing tubules 60–80 microns in diameter and without tunica propria. Tubules are lined with small cells and occasional spermatogonia. A much larger tubular cross-section contains a round body with a core of suspected nuclear origin as depicted. The core is surrounded with concentric rings of material, the outer zone of which stains intensely with the blue of Mallory's trichrome stain. The sustentacular cells of these larger tubules are arranged in two distinct layers, the outer one against the basement membrane, the inner against the round body. Spermatogonia are present. The interstitial tissue is undifferentiated. In other sections an occasional seminal cord was seen.

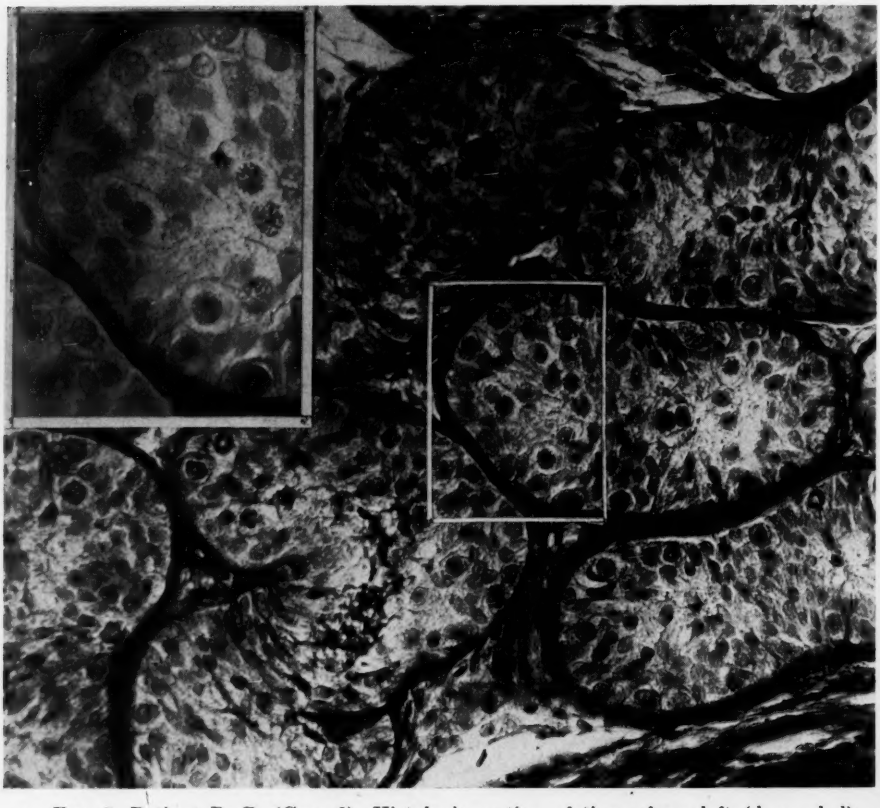

FIG. 5. Patient B. P. (Case 2). Histologic section of tissue from left (descended) testis shows that tubules are larger than those of the right testis and measure 80–120 microns in diameter. They are tortuous, and have lumina and a tunica propria. They contain numerous sustentacular cells and some spermatogonia. Occasional tubules contain, in addition, primary spermatocytes (see insert). The interstitium is not differentiated. (Magnification slightly higher than that of Fig. 3.)

of age. Tempting as it may be to speculate on the hormonal influences which may have stimulated the precocious maturation of the germinal epithelium, two conflicting bits of evidence come to mind. Germ cells in hypophysectomized rats mature to the stages of secondary spermatocytes and spermatids independent of pituitary influence. On the other hand, such maturation has never been observed in the human testis, even in patients 17 to 19 years old with underdeveloped genitalia associated with hypopituitarism. The presence of primary spermatocytes thus may not constitute valid evidence for assuming precocious gonadotropic stimulation of the testes. It is generally believed that the follicle-stimulating hormone (FSH) primarily influences the growth of the seminiferous

tubules and early maturation of the germinal epithelium. If one accepts increases in uterine weight in assay rats as evidence for FSH (our test rats had maximally stimulated uteri!) the explanation of precocious germinal maturation would be obvious. However, for Patient D. H. (Case 1) the histologic responses of the assay-rat ovaries were in the thecal or interstitial elements, suggestive of LH and ICSH. The lack of interstitial-cell differentiation in the descended testes of these 2 patients is in keeping with their young prepubertal body type, and is particularly remarkable since Patient D.H. was excreting gonadotropins of the LH or ICSH type. There is no report available concerning the type of gonadotropic hormone normally excreted in childhood, as pointed out by McArthur *et al.* (3). In a series of 6 boys they found one aged 6 years and one aged 8 years who excreted ICSH, as interpreted by results of the rat ventral-prostate test. They believed that their patients were normal and "exhibited no signs of sexual maturation." Our failure to detect gonadotropin in the urine of Patient B.P. (Case 2) may be attributed to the age of the urine specimens, as they were mailed in for analysis.

There are many case reports of sexual precocity in children with intracranial lesions. These lead to speculations on hypothalamic stimulation of precocious gonadotropic activity of the pituitary. In our 2 cases the skull abnormalities and hydrocephalic changes also invite speculation. For this reason we requested a case summary on the 9-year-old boy with a focal area of precocious germinal cell maturation, included in a series of normal boys reported by Albert *et al.* (4). This boy had a history of vague visual and muscular complaints which merged into a month's illness of vertigo and headache. A craniotomy revealed hydrocephalus due to an inoperable astrocystoma, and the boy died during the first postoperative day. Dr. Albert writes that "this tissue was by some inexplicable error included in a collection of testes of normal boys, and obviously cannot be regarded as such."

We have no explanation for the association of multiple congenital anomalies with precocious maturation of the germinal epithelium in a grossly normal testis.

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FAMILIAL MALE PSEUDOHERMAPHRODISM WITH LABIAL TESTES AND PARTIAL FEMINIZATION: ENDOCRINE STUDIES AND GENETIC ASPECTS

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ABSTRACT

A family is reported in which there were 5 male pseudohermaphrodites in 2 generations. In a given sibling group the males were either all completely normal or all male pseudohermaphrodites. The females were normal. An anti-testis factor, acting in utero to produce male pseudohermaphrodism, and resulting from a dominant heterozygous gene in half of the daughters of affected families has previously been postulated and affords the best explanation of the findings in this family. There was no evidence of endocrine dysfunction in the one male pseudohermaphrodite who was studied. In the 3 who were examined by buccal smear there was no nuclear chromatin. The absence of alkaline phosphatase (histochemical determination) in the testes of one subject was an unexplained and unexpected finding.

WITSCHI, Nelson and Segal (1) in a recent review concluded that a maternal anti-testis factor offered the best explanation for male pseudohermaphrodism. After reviewing 10 families Taillard and Prader (2), however, proposed that inconstant non-disjunction of the X chromosomes was the most likely cause. The family reported here is of particular interest not only in that 5 members in 2 generations were found to be male pseudohermaphrodites, but in that the adults were of an infrequently reported type. The subsequent genetic analysis offers some confirmation of the explanation proposed by Witschi, Nelson and Segal.

CASE REPORTS

The genealogical table of the family is shown in Figure 1.

Case II-4

This patient was referred to the Clinical Center at age 28 for evaluation of her sexual status, in view of a proposed marriage. She was born following a normal 9-month pregnancy and underwent normal growth and development except for sexual structures. She first became aware of abnormal genitalia at age 10, when she noted that she urinated through her vagina. At age 15, according to the patient, female breast development and

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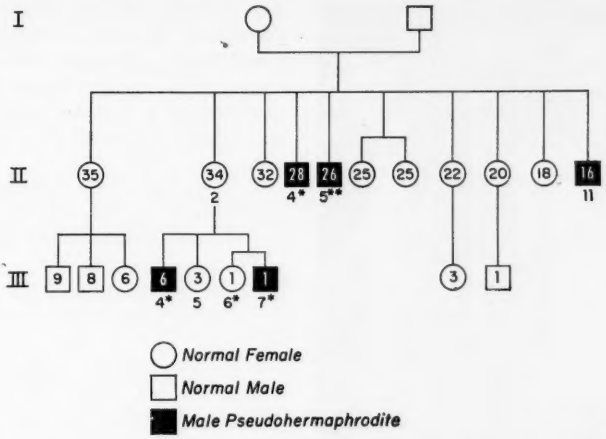

FIG. 1. Genealogical table—male pseudohermaphrodism. The Roman numerals refer to the generation. The patients' ages are within the squares or circles, and their numbers are below the squares or circles. Subject II-4, for example, is a 28-year-old male pseudohermaphrodite. Patients marked by an asterisk (*) were examined by one of the authors (H.L.); a double asterisk indicates that the data were obtained from another hospital. The lack of a normal male sibling in generation II or the family of II-2 is apparent.

a female escutcheon appeared, but on no occasion did she experience any cyclic sensations in her breasts or abdomen or note any bleeding from the genital area. Her interests were always feminine. Female sex orientation occurred at age 15, but she denied attempts at intercourse although she thought she responded normally to erotic stimuli. She had pneumonia four times during childhood but recovered uneventfully. She knew of no symptoms of endocrine disease except as mentioned previously. Her mother had a goiter for many years but had not suffered from any illness and had not required special medication during her many pregnancies.

On physical examination, vital signs were normal, including a blood pressure of 115/70 mg. Hg (standing or supine). A blotchy tan pigmentation over the forehead and malar eminences was suggestive of that seen in Addison's disease. The thyroid showed a slight, diffuse enlargement. The chest and heart were unremarkable except for a grade I apical systolic murmur; neuromuscular and abdominal examinations yielded normal findings. The breasts were of normal size but were puberal in appearance and consistency, with minimal areolar pigmentation, normal nipples and a few periareolar hairs. The height was 70 inches; span, 71 inches; and floor-to-symphysis pubis measurement, 35 inches. The musculature was slightly heavy about the shoulders and thighs, but seemed predominantly female as shown in Figure 2. Body, facial and pubic hair were typically feminine. The clitoris was enlarged, measuring 1.8 cm. in length by 1.5 cm. in diameter, with a series of small blind pores opening on its under surface. Extending posteriorly on either side were 2 somewhat rugose fatty eminences having features both of labia and of a bifid scrotum. A mass measuring 3.5 cm. $\times$ 1.6 cm. could be palpated with ease in each labio-scrotum, and a vas deferens was palpated above each mass. The masses could be moved with ease and pushed up over the symphysis and into the inguinal canal but not into the abdominal cavity. Midway between the labio-scrotal folds there

a b

FIG. 2. Case II-4. a) The slightly heavy musculature about the thighs and shoulders but predominantly female habitus and secondary sex characteristics are apparent. b) The breast development and thin habitus are shown.

was a single opening, and examination through a cystoscope revealed a blind pouch 5 cm. in length with no evident cervix and a urethral orifice opening anteriorly into a normal bladder. No prostatic tissue was noted on rectal examination.

A hemogram and urinalysis findings were normal, as were the levels of serum electrolytes, proteins, blood urea nitrogen and fasting blood sugar. The serum cholesterol level varied between 119 and 135 mg. per 100 ml. before orchiectomy, and between 157 and 180 mg. following orchiectomy. The 24-hour thyroidal I^{131} uptake was 25 per cent; the serum protein-bound iodine level, 5.0 μg. per 100 ml.; and the basal metabolic rate +1 per cent. The serum alkaline phosphatase level was 0.8, 0.9 and 2.0 Besse-Lowry units per 100 ml. (normal 0.8–2.9). Further endocrine studies are reported later in the text. An intravenous pyelogram and roentgenograms of the bones were normal. In a buccal smear, the cellular nuclei were chromatin-negative (male nuclear sex).

At the time of laparotomy a biopsy revealed that there was testicular tissue bilaterally; therefore, both gonads were removed. It was noted that the vasa deferentia coursed normally towards the bladder but did not meet; there was no grossly apparent prostatic tissue. Postoperatively the patient received 5 mg. of diethylstilbestrol daily for two months without effect on her breasts or urogenital sinus. Typical hot flushes occurred when estrogens were discontinued for subsequent plastic repair of her genitalia.

All 11 children in generation II were raised as girls, but 2 were proved to be male pseudohermaphrodites and a third was probably a male pseudohermaphrodite. The occurrence of 2 sets of twins should be noted, particularly the younger pair in which one was a normal female child and the other a male pseudohermaphrodite. The absence of a normal male among 11 children is striking. Two of the 4 children of normal Subject II-2 were similarly affected; again, the absence of a normal male is noteworthy. The

remaining male children in generation III were said to be normal but were not examined. Consanguinity was denied by the family. The genitalia of Subjects I-1 and I-2 were stated to be normal by the family physician.

Case II-5

This patient was the 26-year-old sister of Subject II-4. Her development was identical. Realization of her abnormal genitalia occurred at age 10, followed by an essentially normal female puberty with the development of all secondary female sex characteristics. She remained amenorrheic, however, and at age 20 she presented herself at the University of Virginia Hospital for determination of her sexual status in view of a proposed marriage. She stated that she had had no serious illnesses.

Physical examination revealed a somewhat tall and slender person of feminine appearance, except for slightly heavy musculature about the shoulders and thighs. Blood pressure was 140/80 mg. Hg. The genitalia were similar to her sister's, except that the phallus was larger (3 cm. $\times$ 4 cm.). The gonads were smaller ("peanut sized") but similarly located in the labia. Labia minora were absent. There was a row of small blind pores on the under surface of the phallus (which contained no urethra), and a single small opening between the labio-scrotal folds through which the patient voided. The escutcheon was female, the breasts normal for a woman of 20, and facial hair of normal female type. The remainder of the physical examination yielded normal findings.

At the time of laparotomy, a biopsy showed atrophic testes, which were then removed. No female pelvic structures were seen, and the adrenal areas were normal to palpation. Upon microscopic examination of the atrophic testicular tubules, there was no evidence of spermatogenesis. An intravenous pyelogram was normal and 2 preoperative 17-ketosteroid determinations were reported as 9.8 mg. (creatinine 1.1 Gm.) and 20.5 mg. (creatinine 2.2 Gm.) per twenty-four hours. Postoperatively, gonadotropin excretion was greater than 100 mouse units. The patient was maintained with estrogens, and hot flushes were not troublesome.

Case II-11

This patient, aged 16, and the youngest of the 11 siblings, was stated by the family and the family physician to have genitalia and secondary sex characteristics identical to those of Subjects II-4 and II-5; she had never menstruated. The family felt she should not be subjected to further examination and would permit no studies to be carried out. She was raised as a girl and was in good health.

Cases III, 4-7

Three of the 4 children of Subject II-2, a sister of Subject II-4 were examined in their home by one of us. The findings were as follows:

Case III-4. This eldest child among 4 siblings was raised as a boy and was 6 years of age. He was the product of a normal pregnancy and delivery. The mother denied hormone therapy, endocrine dysfunction and abnormal genitalia, but was not examined. His development and past history were unremarkable.

On examination he appeared to be a normal 6-year-old boy except for the genitalia, which were similar to those of Subjects II-4 and II-5. The phallus was larger proportionally, measuring 1.5 cm. $\times$ 2.0 cm. A row of small blind pores was present on the ventral surface and 2 atrophic labia minora were attached laterally. The presumed

gonads (about 1 cm. in diameter) lay in the suprapubic fat at the upper margin of the labio-scrotal folds, but could easily be brought down into them with gentle pressure. The labio-scrotal folds showed only slight central wrinkling and were more suggestive of labia than of a bifid scrotum. A single small opening was present between the folds, through which the patient urinated. No further examinations were carried out, except a buccal smear in which the cellular nuclei were chromatin-negative.

Case III-7. This child was the youngest of the 4 children of Subject II-2 and was raised as a girl, being 13 months old at the time of examination. She was one of fraternal twins, born of a pregnancy during which the mother experienced marked edema of the ankles and some hypertension in the last trimester, and mild nausea in the first trimester. Information concerning the placental relationship between the fetuses was not available. *The twin sister* (*III-6*) was perfectly normal both by history and a careful physical examination. Both were 31 inches in length, but the normal sister was definitely more obese. Subject III-7's development was normal, and she suffered only a few respiratory infections.

Physical examination revealed nothing abnormal except for the genitalia. The labia appeared relatively normal, with only suggestive central wrinkling. No gonads could be palpated in the labia or in the suprapubic or inguinal area. Again, there was a row of pores on the under surface of the phallus through which the patient voided. Upon rectal examination there was no evidence of female pelvic structures or prostatic tissue. In a buccal smear all the cellular nuclei were chromatin-negative. The family decided against any further studies.

Case III-5. This child was not examined, but was said to be a normal female.

HORMONE STUDIES

Methods

Urinary 17-ketosteroids were determined by the method of Callow, Callow and Emmens (3) (normal range for adult males, 6–16 mg. per twenty-four hours; and for adult females, 4–14 mg.). Levels of 17,21-dihydroxycorticoids in the urine following β-glucuronidase hydrolysis (normal range, 5–12 mg. per twenty-four hours) and in the blood (normal range, 5–20 μg. per 100 ml.) were measured by Peterson's modification of the Silber-Porter method (4), and urinary gonadotropin by the method of Klinefelter *et al.* (5). Buccal smears were made and examined for the presence of chromatin in the cellular nuclei, as described by Marberger, Boccabella and Nelson (6). Adrenal function tests were carried out by administration of 40 units of ACTH intravenously over an eight-hour period. Stimulation with gonadotropin consisted of the administration of 5000 units of chorionic gonadotropin (Ayerst) intramuscularly each day for seven days.

Results in Case II-4

The 24-hour urinary 17-ketosteroid output ranged between 8 and 13 mg. initially. After orchiectomy the average output decreased by 4–5 mg.

(Fig. 3). The response to ACTH was normal both before and after operation. Preoperatively, following stimulation with gonadotropin, there was a rise in 17-ketosteroid excretion to 16–19 mg. per twenty-four hours, but no rise occurred with identical stimulation after orchiectomy. Blood and urinary corticoid values were normal, as was the response to ACTH. There was no change following orchiectomy.

The urinary gonadotropin titer was initially less than 10 mouse units per twenty-four hours, and rose to 91 and 134 mouse units five months after orchiectomy.

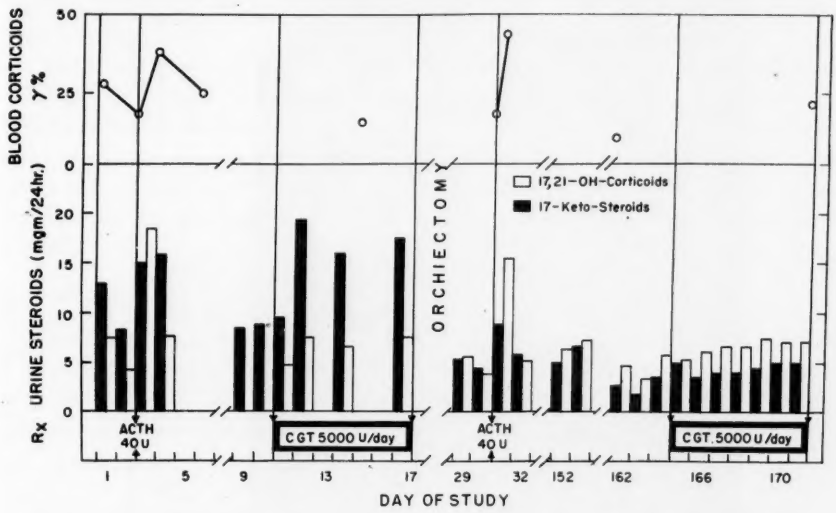

FIG. 3. Steroid data. Blood and urinary 17,21-dihydroxycorticoid levels were normal throughout, and were unchanged by orchiectomy. There was a significant drop in 17-ketosteroid excretion following orchiectomy and no response to stimulation with chorionic gonadotropin, in contrast to the initial normal response. The response to ACTH was considered normal in both instances.

HISTOLOGY OF THE GONADS

Case II-4

Microscopic examination of the testes revealed a marked degree of Leydig-cell hyperplasia and varying degrees of tubular atrophy (Fig. 4, a and b). In some areas the Leydig cells formed compact masses with only a light stroma and staining was markedly hyperchromatic and basophilic. The majority of these cells showed only a slight or a negative reaction for ascorbic acid, cholesterol and lipids, indicating diminished functioning of these cells. Abundant brown granules were seen, however, which were

FIG. 4. Microscopic view of sections from testes: **a)** Case II-4, showing an area of tubular atrophy with some persisting Sertoil cells and interstitial-cell hyperplasia. **b)** View of adjacent area in Case II-4, showing spermatogenesis and interstitial-cell hyperplasia. **c)** Case II-5; characteristic view showing atrophic tubules and interstitial-cell hyperplasia. No spermatogenesis was seen, in contrast to the pattern in Case II-4. (×75).

metachromatic, Schiff-positive and resisted digestion by Taka diastase and hyaluronidase. These granules were probably lipofuscins. Some of the Leydig cells had normal morphologic and histochemical characteristics. In the less compact areas of Leydig cells, the cells were surrounded by a more dense stroma and showed little evidence of functional activity.

Repeated attempts to stain the testes and epididymis for alkaline phosphatase (7) yielded negative or only faintly positive results, except for the blood vessels which reacted normally. Simultaneously treated normal testicular tissue showed the expected positive staining for alkaline phosphatase in the nucleus of the Sertoli cells, spermatogonia, spermatocytes, connective tissue cells, and Leydig cells as well as in the vessels and basal membranes.

The tubules varied greatly in appearance. In some areas there was only a slight increase in the number and thickness of the reticular fibers, with nearly normal spermatogenesis (Fig. 4b). In other areas, however, there was marked collagen sclerosis and hyalinization, no spermatogenesis, and only a few Sertoli cells (Fig. 4a). Such contrasting tubules were often adjacent to each other. The epididymis, rete testis and vas deferens were normal.

Case II-5

The testes in this case were similar to those in Case II-4, but spermatogenesis was not noted. The Leydig-cell hyperplasia was more marked, as was the tubular sclerosis (Fig. 4c). No tissue was available for alkaline phosphatase or other special stains.

DISCUSSION

The family reported here included 5 male pseudohermaphrodites. In the 2 sibling-groups which were studied, all males were pseudohermaphrodites; in the other groups, all were reported as normal by the family. The pattern of abnormalities was remarkably constant considering the variation in age; each affected male had an enlarged clitoris, labia with varying scrotal characteristics, and a urogenital sinus containing the urethra. In the 2 adults who were studied there were histologically proven testes, female breast development, female hair distribution, and labial testes. In the 2 affected children who were studied the testes had almost descended to the labia in the older child, and were not palpable in the younger. It seems probable, in view of their similarity to the older members of the family, that they will undergo a female puberty with further descent of the testes and further distention of the labia. In the 3 subjects available for buccal smear studies, the cellular nuclei were chromatin-negative.

Two similar cases were reported by Witschi and Mengert (8). In these

cases there was also marked feminization in the presence of labial testes and spermatogenesis, but the urethra opened externally rather than through a urogenital sinus. There were also reportedly 2 normal males in the same sibling group, one of whom had a son.

The endocrine studies carried out on Subject II-4 are remarkable primarily for the lack of significant abnormalities. There was no evidence of adrenal dysfunction and the responses to ACTH and gonadotropin stimulation were normal. The drop in 17-ketosteroid excretion after orchiectomy as well as the occurrence of hot flushes and lack of response to gonadotropin were definite, although indirect, indications of the endocrine activity of the testes. No estimate of the androgen content of the urine was made. The mechanism of feminization in this case is not explained by these studies.

The marked Leydig-cell hyperplasia noted in Cases II-4 and II-5 is characteristic of male pseudohermaphrodism, but in Case II-4 may have been due in part to the administration of gonadotropin (9). The degree of spermatogenesis present in some tubules in Case II-4 is uncommon, but the vas deferens ended blindly in both subjects and neither would have been fertile. It is of interest that although the hormone studies on urine and blood showed normal levels of steroids and gonadotropin, the histochemical studies on the testes showed immaturity and diminished function. It is possible that this reflects diminished androgen production, but there is no direct evidence to support this idea. There was no apparent relationship between the staining qualities of the Leydig cells and the degree of spermatogenesis in adjacent tubules. The absence of alkaline phosphatase, as shown by lack of staining in the testes of Subject II-4 was an unexpected finding.

Among the siblings of the 10 families they reviewed, Taillard and Prader (2) found a proportion of 9 females to 7 male pseudohermaphrodites to 2 normal males. They noted that if there was non-disjunction of the X chromosomes in 7 out of 8 ova, the offspring would occur in the ratio of 7xxx: 7xxy:2xx:2xy (where xxx represents super-females and xxy represents male pseudohermaphrodism). They proposed this interpretation as the best explanation of male pseudohermaphrodism. They did not, however, carry their analysis beyond the first generation. In the present family, 6 of 7 married daughters had children, and most would be xxx. Presumably, the xxx females would all have carrier daughters or male pseudohermaphrodites, as well as a portion of the xx females. This would result in a much higher ratio of carrier to normal daughters in the second generation than the 1:1 ratio seen in the family reported by Pettersson and Bonnier (10), and an even lower ratio in the present family. Moreover, the absence of any normal male children in the 2 affected sibling groups (15 children) is unexplained,

except as a somewhat unlikely chance. In addition, although Danon and Sachs (11) found more than the normal male number of chromatin-positive cells in the patients with male pseudohermaphrodism whom they postulated to be xxy, the 3 male pseudohermaphrodites reported here did not have high counts; in fact, no chromatin-positive cells were noted. Chu and Giles (12) carried out a complete chromosome analysis in a male pseudohermaphrodite and found a normal chromosome complement. There seems, therefore, little to support such an explanation in this and some other families. In several of the families reviewed by Taillard and Prader, however, there were normal males as well as a high proportion of carrier females in the affected sibling groups, and in these families the explanation may be correct, although the exact chromosome counts are not available. It may well be that there are multiple causes of male pseudohermaphrodism and that it is a mistake to attribute a common etiology to the clinical spectrum extending from hypospadias to phenotypic females with testes.

Witschi, Nelson and Segal (1) in a recent discussion of this problem concluded that a maternal anti-testis factor transmitted in utero to all male fetuses offered the best explanation of the genetic behavior of these families. This factor, resulting from a dominant trait in a heterozygous mother would also be passed on to half of her daughters. This explanation also affords an adequate explanation for the family reported here. No information about the nature of the anti-testis effect was obtained from this family. In 1942, Witschi and Mengert (8) suggested studies of known transmitting mothers, but to date none have been reported. They remain of critical importance if efforts are to be made to prevent the occurrence of additional male pseudohermaphrodites in such families.

Acknowledgment

The authors would like to thank Dr. Roy Hertz for his many valuable suggestions, and to acknowledge the able help of Dr. Walter Heston in interpreting the genetics, and of Dr. Warren Nelson in reviewing the buccal smears. The excellent laboratory work of Mr. Donald Spencer, Mr. Silas Jackson, Mr. Foster Burnett and Mrs. W. Linell Murphy was invaluable in carrying out these studies.

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STRUMA LYMPHOMATOSA: A REPORT OF THREE CASES IN ONE FAMILY

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ABSTRACT

Three cases of struma lymphomatosa (Hashimoto type) in members of one family are presented. Such a finding has apparently not been reported previously. These cases are of further interest in that they all occurred in young girls and in one instance the process recurred in identical form eight years later.

THE purpose of this paper is to present 3 cases of struma lymphomatosa in one family, an occurrence apparently not previously reported. These patients, 2 sisters and a paternal first cousin, were young girls who lived near each other.

CASE HISTORIES

Case 1

M.E.T., a 22-year-old white female, was seen in 1953 because of a recurrent goiter. In 1945, at 14 years of age she had had a subtotal thyroidectomy and one year later noted a recurrence of the thyroid enlargement. Examination of the records of that operation showed that 34 grams of thyroid tissue had been removed. Physical examination on her second admission showed that the right thyroid lobe was enlarged to three times the normal size, and the left to about twice the normal size. No signs of hypo- or hyperthyroidism were detected. A subtotal thyroidectomy was again carried out and 37 grams of thyroid tissue removed.

Grossly the tissue removed at the time of the second procedure was tan to pink in color, nodular in configuration, and firm in consistency. On microscopic examination the picture was mainly one of numerous lymphoid patches with prominent germinal centers. The connective-tissue stroma showed focal collections of lymphocytes and plasma cells. The acini were small and the epithelium acidophilic (Fig. 1).

The histologic appearance of the tissues removed at the two operations was strikingly similar. Figure 2 is a photomicrograph of tissue removed at the first operation. Both specimens were diagnosed as struma lymphomatosa (Hashimoto type) (1).

An agar diffusion antibody test for struma lymphomatosa carried out five years after surgery yielded negative results (2).

Case 2

P.B.T., an 18-year-old white girl and sister of M.E.T., was seen because of a mass in the neck of nine years' duration. The mass was a diffusely enlarged thyroid gland,

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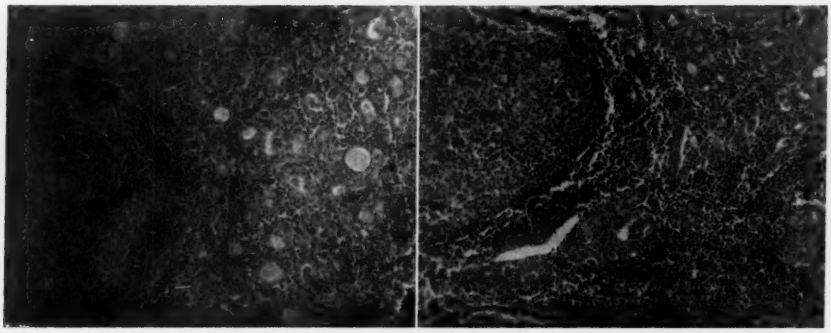

1 2

FIG. 1. *Case 1.* Photomicrograph of thyroid tissue removed at second operation in 1953. (×67)

FIG. 2. *Case 1.* Microscopic appearance of thyroid tissue removed at first operation in 1945. (×67)

enlarged to about four times normal size. The patient was euthyroid. The weight of the thyroid tissue removed by subtotal thyroidectomy was 101 grams.

By gross examination the thyroid tissue was gray-pink in color, with accentuated nodules separated by gray-white trabeculae. The histologic pattern was characterized by widely scattered small acini and variable quantities of lymphocytic infiltration. In some areas there was distinct lymph follicle formation. Many of the cells lining the small acini were relatively large and contained granular oxyphilic cytoplasm (Fig. 3). The diagnosis was struma lymphomatosa (1).

Four years after operation the Ouchterlony agar diffusion test yielded negative results (2).

There were 5 siblings in the family of these 2 girls, but only the patients reported here had ever had goiters.

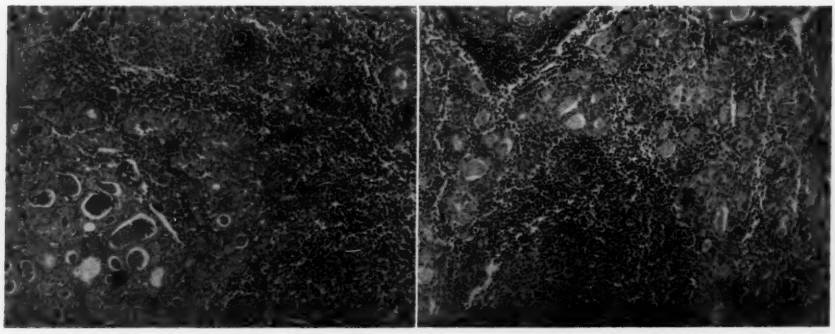

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FIG. 3. *Case 2.* Microscopic appearance of thyroid tissue. (×67)

FIG. 4. *Case 3.* Photomicrograph of thyroid tissue. (×67)

Case 3

M.F.T. was a 19-year-old white female and a paternal first cousin to M.E.T. and P.B.T. She had had a mass in her neck for three years with a sensation of choking for one year. Both lobes and the isthmus of the thyroid gland were diffusely enlarged to twice normal size. The patient was euthyroid. At operation, 15.2 grams of thyroid tissue were removed in a limited subtotal resection.

The specimen of thyroid, grossly, was yellow to gray in color with streaks of pink tissue coursing through it. Microscopic examination showed an intense lymphocytic infiltration, with formation of follicles in some areas. The cells lining the acini were low, cuboidal and eosinophilic-staining (Fig. 4). The diagnosis was struma lymphomatosa (Hashimoto type) (1).

An Ouchterlony diffusion test four years after thyroidectomy yielded negative results. No other person in this girl's immediate family ever had a goiter.

DISCUSSION

The interesting type of thyroiditis reported here was first described by Hashimoto (3) in 1912, when he presented 4 cases of what he considered to be a new type of thyroiditis. Subsequently, this clinico-pathologic process became commonly known as "Hashimoto's disease." The distinctiveness of the abnormality was re-emphasized in 1931 by Graham and McCullagh (4) when they reported 4 additional cases of struma lymphomatosa and separated Hashimoto's disease once and for all from the other types of thyroiditis. Since that time over 1,000 cases of struma lymphomatosa have been reported (5).

The area in North Carolina in which the girls of this study were reared is not considered an endemic goiter area (6).

In earlier reports (7–12), Hashimoto's disease was characterized as a finding in the thyroid gland of certain middle-aged females but the number of cases observed in younger patients is increasing (13–25).

In the first case reported here, struma lymphomatosa recurred in a form histologically identical to that seen in the tissue removed eight years earlier. Re-operations necessitated by the enlargement of a lobe not resected at the original operation have been reported (11, 15, 26, 27). Also, although somewhat more unusual (17), the recurrence of Hashimoto's struma has been reported after bilateral subtotal thyroidectomy (14, 20).

On microscopic examination all 4 specimens (2 from the first case and 1 each from the other 2 cases) showed oxphilia of the acinar epithelium. This feature, together with the histologic findings already described, indicate that these are "classic" examples of struma lymphomatosa (Hashimoto type) (13, 14, 28, 29). Many cases reported in younger people have not exhibited this histopathologic feature (17, 19, 23, 28).

Acknowledgments

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FACTORS INFLUENCING TAURINE EXCRETION IN HUMAN SUBJECTS*

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ABSTRACT

The average 24-hour urinary excretion of taurine in a normal male during a period of ordinary dietary intake was 161 mg. During a period of high protein intake, the daily excretion of taurine (193 mg.) was not significantly affected. Normal values in 20 adult women and in 20 preadolescent children of both sexes are also presented. In 6 patients with known or suspected adrenocortical disorders, taurine excretion was sharply increased following treatment with ACTH or 9α-fluorohydrocortisone. No correlation between taurine excretion and chronic disease was found. The data suggest that there is a relationship between adrenocortical function and taurine excretion.

INTRODUCTION

IT HAS been shown previously (1) that in adrenalectomized rats the daily excretion of taurine is only half that observed in animals subjected to a sham operation. Irradiated adrenalectomized rats, however, excrete twice as much taurine following exposure to 400 rads as do sham-operation irradiated control animals. These observations suggest that absence of adrenal hormones is followed by changes in the metabolism of sulfur-containing amino acids, of which taurine is an excretory end-product (2–4). Prior removal of the thymus and spleen from rats which subsequently received 400 rads of total body x-irradiation did not reduce the amount of taurine excreted by these animals. This observation made it appear unlikely that the increase in taurine excretion simply represents increased breakdown of tissue, as lymphoid tissue is readily destroyed by exposure to irradiation (5).

Since hormones of the adrenal gland appear to influence taurine excretion in the rat, it was of interest to examine this relationship in normal and pathologic human subjects.

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CLINICAL MATERIAL AND METHODS

To obtain knowledge of the daily variation in taurine excretion under conditions that resembled those that might occur in day-to-day living, a normal male subject was observed under continuous experimental conditions. For the first five days he was instructed to eat as he usually did, except that on the third day he was to consume generous quantities of alcohol in any form he chose. Since proteins are high in sulfur-containing amino acids it was necessary to determine whether a high protein intake would significantly alter taurine excretion. Therefore, from the sixth to the thirteenth day of the study he was instructed to eat as much protein as he could tolerate. Food records were kept during this period and approximately 18–22 per cent of the caloric intake consisted of protein. The caloric intake varied from 2300 to 3000 calories. It was thought that the foregoing conditions simulated those which might obtain in patients whose complete caloric, alcoholic and dietary history was not known.

The children from whom urine specimens were obtained were all from doctors' families and were ostensibly in good health. Care and effort were exercised to insure completeness of collection.

Urine was collected in 24-hour specimens under refrigeration from patients who had known or suspected adrenocortical disease. Urine specimens from normal control subjects and from patients who were not being studied for excretion of adrenocortical metabolites were collected under toluene.

Patients were not specifically selected for hospital care for the purposes of this study but were hospitalized for conditions related to the primary diagnosis. Those with chronic diseases received only salicylates and hypnotics such as barbiturates and chloral hydrate during the period of study. In none of the cases listed in Table 3 had hormones been administered for at least two weeks prior to the diagnostic procedures outlined.

Taurine analyses were carried out by the method of Pentz *et al.* (6); urinary 17-hydroxycorticosteroids were determined by the method of Silber and Porter (7), and 17-ketosteroids by a proposed standard method (8). Creatinine was determined by the Jaffe reaction (9), and uric acid by the direct method of Folin (10).

RESULTS

Table 1 shows the results obtained on a normal male under continuous experimental conditions. The average taurine/creatinine ratio (0.83) during the eight days of high protein intake was not significantly different from that (0.86) during the period of normal protein intake. During the period when protein consumption was approximately twice normal, slightly higher taurine values were obtained (average, 193 mg.) than during the baseline period (average, 161 mg.) but the higher values were well within the normal range (1). Having established that rather large variations in the dietary component deemed most likely to alter taurine excretion, namely protein, did not produce large variations in taurine excretion, further investigations were made in normal human subjects.

In a series of 20 apparently normal women between the ages of 17 and 54 years, taurine excretion ranged from 115 to 331 mg. per twenty-four hours, with an average value of 196 mg. Table 2 shows the values obtained in preadolescent children; these always proved to be somewhat lower than

TABLE 1. EFFECT OF HIGH PROTEIN DIET ON TAURINE EXCRETION IN A NORMAL MALE

(White male, aged 34; weight 145 pounds.)

Day	Urine volume (ml.)	Creatinine (Gm./24 hrs.)	Uric acid (Gm./24 hrs.)	Taurine (mg./24 hrs.)	Ratio, mg. taurine / Gm. creatinine
1	1,100	1.77	0.478	132	75
2	1,650	2.07	0.742	187	90
3	3,700	2.00	0.540	236*	118*
4	1,500	1.98	0.540	160	84
5	2,430	1.73	0.462	164	95
				Avge. 161	*Avge. 86*
6 H.P.**	1,350	2.04	0.680	207	101
7 H.P.	1,700	2.12	0.850	201	95
8 H.P.	3,100	2.35	0.576	199	85
9 H.P.	2,000	2.32	0.752	206	89
10 H.P.	1,800	2.25	0.594	170	75
11 H.P.	2,425	2.61	0.540	187	72
12 H.P.	2,700	2.43	0.772	173	71
13 H.P.	2,400	2.59	0.758	203	78
				Avge. 193	*Avge. 83*

* During a day of high alcohol consumption. Not included in the average.
** H.P. = High protein diet.

in adults, and there was no apparent difference between the sexes.

Table 3 shows the data on patients who had known or suspected adrenocortical disease and who received ACTH or 9α-fluorohydrocortisone for diagnostic purposes. It is evident that there was a correlation between the excretion of adrenocortical metabolites and that of taurine. Prior to administration of hormone, taurine excretion was low in all but 1 case. These low values occurred sufficiently frequently in diseases related to adrenocortical malfunction to suggest that specific relationships may be established in the future.

Since the patients in Table 3 had chronic diseases that might reflect derangements in physiology simply because of their chronicity, it was desirable to evaluate patients with other chronic illnesses in respect to taurine excretion. Thus the patients in Table 4 had diseases which had been present from two to many years. At the time of these observations no patient had received or was receiving corticosteroid therapy. It may be seen that taurine excretion values ranged from 55 mg. to 497 mg. per twenty-

TABLE 2. TAURINE EXCRETION IN PREADOLESCENT CHILDREN

Subject			Urine volume (ml.)	Taurine (mg./24 hrs.)
Initials	Sex & Age (yrs.)			
J.M.	M	4½	480	94
G.M.	M	4½	480	57
B.M.	M	7	600	111
L.K.	M	8	410	75
L.K.	M	7	505	86
L.R.	M	7	650	127
L.S.	M	7	500	41
B.P.	M	5½	550	50
D.P.	M	10	550	74
S.T.	M	10	700	92
G.T.	M	6	400	58
E.C.	F	8	400	104
L.M.	F	9	800	111
A.M.	F	11	1,150	103
B.C.	F	7	550	61
M.H.	F	8	330	73
C.S.	F	11	550	112
J.S.	F	8	800	70
M.C.	F	10	650	72
B.T.	F	4	450	50

four hours, with no consistent pattern that might be attributed to chronicity of illness.

Table 5 shows the taurine excretion data on a patient with rheumatoid arthritis who had been maintained with prednisone, receiving at least 5 mg. daily for months prior to this series. The correlation between decreasing amounts of prednisone and increasing taurine excretion is striking.

DISCUSSION

Normal values for the excretion of taurine in men have been reported by Stein (11) (column chromatography) and by Pentz *et al.* (6) using a chemical method which includes the small amount of cysteic acid excreted. The values reported by Stein in 8 normal males ranged between 86 and 294 mg. per twenty-four hours. In the series of 19 males reported by Pentz *et al.*, the taurine content of 24-hour urine specimens not subjected to hydrolysis was between 44 and 514 mg. However, with the exception of the 2 extreme values, the 17 other determinations were between 120 and 287 mg. When these analyses were made there was no obvious reason for excluding the 2 outside values and they were accordingly reported. The

TABLE 3. TAURINE EXCRETION IN PATIENTS WITH KNOWN OR SUSPECTED ADRENOCORTICAL DISEASE; EFFECT OF ADMINISTRATION OF HORMONE

Patient	Urine volume (ml.)	Creatinine (Gm./24 hrs.)	Uric acid (Gm./24 hrs.)	17-Keto-steroids (mg./24 hrs.)	17-OH-steroids (mg./24 hrs.)	Taurine (mg./24 hrs.)	Dates	Hormone admin'd
C.G., white female aged 15, wt. 129 lbs.	1000	0.59	0.130	7.2	3.1	88	Mar. 13–14	
							Mar. 14	25 u. ACTH*
Idiopathic hirsutism	1200	0.46	0.252	25.8	13.9	130	Mar. 15–16	25 u. ACTH
	1000	0.73	0.144	37.0	20.0	177	Mar. 16–17	
C.E., colored male aged 58, wt. 139 lbs.	2200	1.23	0.220	10.4	4.5	71	Apr. 7–8	
	2000	1.06	0.160	10.6	3.5	64	Apr. 8–9	
							Apr. 9	40 u. ACTH
Multiple myeloma; Klinefelter's syndrome	2800	1.30	0.292	37.7	22.2	143	Apr. 10–11	40 u. ACTH
	2500	1.70	0.166	41.2	28.7	122	Apr. 11–12	40 u. ACTH
	2200	1.50	0.264	15.8	5.3	119	Apr. 12–13	
E.K., white female aged 41, wt. 115 lbs.	1000	0.59	0.170	11.7	3.7	73	Apr. 11–12	
	1000	0.60	0.196	5.8	5.0	78	Apr. 13–14	
							Apr. 14	25 u. ACTH
Hyperthyroidism	1200	0.69	0.644	11.5	11.8	245	Apr. 14–15	
	1000	0.62	0.310	5.6	3.9	93	Apr. 15–16	
M.P., white female aged 58, wt. 170 lbs.	1800	0.65	0.064	17.6	9.9	176	Apr. 9–10	
	1600	0.77	0.136	19.7	9.5	173	Apr. 10–11	
	2000	0.72	0.056	26.8	13.0	230	Apr. 11–12	9α-FHC,** 7.5 mg.
Diabetes mell.; ess. hypertension hirsutism	2600	0.68	0.046	13.3	8.3	300	Apr. 12–13	9α-FHC, 10 mg.
	2200	0.89	0.374	7.2	1.7	283	Apr. 13–14	9α-FHC, 10 mg.
	2400	0.80	0.044	12.2	1.5	245	Apr. 14–15	9α-FHC, 7.5 mg.
B.R., white female aged 20, wt. 180 lbs.	1000	1.14	0.176	14.3	4.2	78	Apr. 15–16	
							Apr. 17	25 u. ACTH
	1500	0.84	0.554	34.0	28.0	185	Apr. 17–18	
Idiopathic hirsutism	900	0.64	0.252	40.0	10.0	124	Apr. 18–19	25 u. ACTH
	1000	1.27	0.026	17.3	5.2	73	Apr. 19–20	9α-FHC, 10 mg.
	1200	1.30	0.600	9.2	1.5	125	Apr. 20–21	9α-FHC, 10 mg.
M.S., white female aged 40, wt. 173 lbs.	1400	0.70	0.356	20.1	3.3	61	Mar. 3–4	
							Mar. 7	25 u. ACTH
Adrenocort. hyperfunction	3000	1.20	0.794	70.0	42.3	203	Mar. 7–8	

* In all cases ACTH was given by slow intravenous infusion and subsequent to the collection of urine.

** 9α-Fluorohydrocortisone.

TABLE 4. TAURINE EXCRETION IN PATIENTS WITH CHRONIC DISEASES; NO CORTICOSTEROID THERAPY

Diagnosis	Patient: Age—Sex—Weight	Urine volume (ml.)	Taurine (mg./24 hrs.)
A. Medium Range of Taurine Excretion (115–330 mg.)			
1. Gout (tophaceous)	43—M—200	2,680	135
2. Hodgkin's disease	43—M—124	2,000	229
3. Rheumatoid arthritis with hypothyroidism	72—F —149	2,600	228
4. Lymphosarcoma	33—M—175	2,980	220
5. Diabetes mellitus with chromophobe adenoma	62—F —167	2,800	194
6. Rheumatoid arthritis	32—M—160	1,400	236
7. Gout (non-tophaceous)	65—M—190	2,220	172
8. Polycythemia	58—F —190	2,000	118
9. Rheumatoid arthritis	48—F —150	1,400	261
10. Rheumatoid arthritis	53—M—158	2,640	214
B. High Taurine Excretion (more than 330 mg.)			
1. Hodgkin's disease	26—M—150	1,990	408
2. Diabetes insipidus	30—F —130	9,200	497
C. Low Taurine Excretion (less than 115 mg.)			
1. Polycythemia	50—F —150	1,500	76
2. Rheumatoid arthritis	30—F —105	500	104
3. Gout (tophaceous)	51—M—208	2,000	82
4. Diabetes mellitus with multiple myeloma	60—F —145	1,300	55
5. Diabetes mellitus	52—F —140	925	95
6. Sjögren's syndrome	64—F —110	1,200	58

low value of 44 mg. was obtained on the urine of a young man who was underweight but who, with the exception of some previous cutaneous disturbances, appeared to be in good health. The high value of 514 mg. was obtained on the urine of a physician who presented no abnormality to account for it. In view of our current findings, it may be that these 2 values should not have been included in the normal series.

The unexpected increase in taurine excretion which accompanied the high intake of alcohol (Table 1) is noteworthy. Both Smith (12) and Forbes and Duncan (13) showed that following the administration of alcohol the amount of cholesterol and ascorbic acid in the adrenals is decreased in both rats and guinea pigs. These findings are believed to reflect the release of adrenocortical hormones. It remains for subsequent research to examine the possibility of a more direct link between sulfur and alcohol metabolism.

Examination of the data in Table 3 shows that administration of ACTH alone was always promptly followed by a sharp rise in the excretion of

taurine. Similarly, when 9α-fluorohydrocortisone was given alone, there was a rise in the excretion of taurine. Since ACTH is known to be an effective liberator of endogenous glucocorticoids, and since (at the dosage levels used, 9α-fluorohydrocortisone is a potent glucocorticoid, these data suggest that hormones having glucorticoid activity tend to raise the level of taurine excretion.

On first inspection the data listed on the case of rheumatoid arthritis in Table 5 might appear to be contradictory. However, prednisone is

TABLE 5. TAURINE EXCRETION IN A CASE OF RHEUMATOID ARTHRITIS MAINTAINED WITH PREDNISONE

(J.K., white female, aged 15; wt. 114 lbs.)

Volume of urine (ml.)	Creatinine (Gm./24 hrs.)	Uric acid (Gm./24 hrs.)	Dates	Taurine (mg./24 hrs.)	Prednisone therapy (mg./day)
550	1.08	0.292	Mar. 20 to 21	92	4
400	0.82	0.242	Mar. 21 to 22	62	4
450	0.90	0.306	Mar. 22 to 23	63	3
			Mar. 23		3
450	0.72	0.262	Mar. 24 to 25	66	3
450	0.88	0.232	Mar. 25 to 26	62	3
550	0.88	0.278	Mar. 26 to 27	75	3
550	Not det'd	Not det'd	Mar. 27 to 28	152	2
800	0.76	Not det'd	Mar. 28 to 29	203	2
600	0.76	0.268	Mar. 29 to 30	152	1
			Mar. 30		1
1,350	0.92	0.528	Mar 31 to Apr. 1	140	1
1,350	Not det'd	0.614	Apr. 1 to 2	174	1
1,150	0.86	0.760	Apr. 2 to 3	213	0
750	Not det'd	Not det'd	Apr. 3 to 4	248	0

known to be a potent inhibitor of the release of endogenous ACTH (14) when administered at somewhat higher dosage levels than shown in Table 5. Although dosages of prednisone in the present instance were low, treatment had been continued for some months. It seems probable that normal release of endogenous adrenocorticotropin may have been inhibited. Thus, a condition of restricted endogenous supply of glucocorticoids would not be surprising. It is suggested that the rise in taurine excretion which followed the gradual withdrawal of prednisone reflected the return to more active pituitary function.

Chronic disease may or may not influence the level of adrenocortical activity. Since no evaluation of the functional level of the adrenal cortices was attempted in the chronically ill patients represented in Table 4, no

correlation between taurine excretion and cortical activity could be attempted. However, within the disease groups so far studied, no regular pattern of taurine excretion has been observed, with the exception that low values have appeared more often among patients suspected of or known to have adrenocortical malfunction. Further work is needed to clarify the relationship which is suggested by the present data.

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FUNCTIONING ISLET-CELL CARCINOMA OF THE PANCREAS WITH CUSHING'S SYNDROME

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ABSTRACT

An unusual case of Cushing's syndrome associated with a functioning islet-cell carcinoma of the pancreas in a 36-year-old woman is presented. The patient had the moon facies, hypertension and striae of Cushing's syndrome, with a right upper abdominal mass and an elevated 17-ketosteroid excretion of 28.1 mg. per twenty-four hours. At operation a large mass was found, which at the time was presumed to be of right adrenal origin; local and hepatic metastases were present. Biopsy of the mass revealed poorly differentiated carcinoma. Postoperatively hypoglycemia developed and progressed in severity to the point of constant convulsions and death in spite of large amounts of glucose orally and intravenously. During this postoperative period, the urinary excretion of adrenal steroids increased markedly and the blood insulin level was five to ten times the normal value. Instead of adrenal carcinoma, marked adrenal hyperplasia was found at necropsy, associated with an islet-cell carcinoma of the pancreas.

THE occurrence of adrenocortical hyperfunction, or Cushing's syndrome, in association with a functioning islet-cell carcinoma of the pancreas has not previously been reported to our knowledge.

In 1946 Crooke (1) reviewed 4 cases of Cushing's syndrome with acinar carcinoma of the pancreas. Since then del Castillo *et al.* (2) and Rosenberg (3) each reported a case of islet-cell carcinoma with basophilism. All 6 patients had nonfunctioning pancreatic lesions. In addition to the rare pancreatic disorders reported in association with Cushing's syndrome, adrenal abnormalities have infrequently been noted in cases of hyperinsulinism. A review of 398 cases of islet-cell tumors of the pancreas by Howard *et al.* (4) mentions the finding of pituitary basophilism at autopsy only in 2 cases with hyperinsulinism. One patient with a nonfunctioning pancreatic lesion was found to have adrenocortical hyperplasia at necropsy. The cases of multiple endocrine adenomas reported by Underdahl (5) and Moldawer (6) and their associates include 7 with adrenal nodules or gross adrenal

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enlargement. However, in none of the foregoing cases was there clinical evidence of adrenal hyperfunction.

The present case is of particular interest because of the development of constant hypoglycemia in spite of excessive amounts of corticosteroid production by the adrenals. Although the severe hypoglycemia suggests the presence of excess insulin of pancreatic origin, it is noteworthy that hypoglycemia has been reported in association with nonpancreatic tumors and normal histology of the pancreas by Silvis and Simon (7), Scholtz *et al.* (8) and Staffieri *et al.* (9). However, we found increased amounts of circulating insulin-like activity in this patient and believe there was clinical and pathologic evidence of simultaneous hyperfunction of the pancreas and adrenals.

CASE REPORT

A 36-year-old white female was admitted to the Bryn Mawr Hospital March 20, 1957, because of fullness of the face, moderate hirsutism and gain in weight of two months' duration. Oligomenorrhea had been present for six months before hospitalization. There was no history of visual disturbance, and no prior cardiorenal or endocrine disturbance. The patient had had 4 normal pregnancies. Review of systems was otherwise unremarkable.

The pertinent findings on initial physical examination included roundness of the face and a moderate amount of fine hair on the upper lip and cheeks. The thyroid gland was not enlarged. Blood pressure on admission was 140/90 mm. Hg, with subsequent variations between 120/82 and 152/90. There was no cardiac enlargement. The breasts were relatively small and flat. In the abdomen a right upper-quadrant mass about 12 cm. in diameter was palpated. This appeared to involve the right kidney and right lobe of the liver and could be felt from front to back. No ascites or purplish striae were noted. The clitoris was not enlarged.

Laboratory and x-ray findings: The routine laboratory findings are summarized in Table 1. X-ray examination showed a normal skull, sella turcica, and chest. An intravenous urogram was suggestive of a mass above the right renal shadow. An air study was not performed.

Clinical course: Because of the physical findings suggestive of Cushing's syndrome, the large abdominal mass in the right upper quadrant, and the elevated urinary 17-ketosteroids, the patient was scheduled for laparotomy with a preoperative diagnosis of right adrenal carcinoma.

The laparotomy was performed on March 27, 1957, one week after admission. An extensively infiltrating tumor involving the liver and adjacent lymph nodes was found. The right adrenal gland could not be visualized, but was palpably enlarged by tumor. Biopsy of the liver and lymph nodes revealed poorly differentiated carcinoma, not diagnostic of a primary site of origin.

On the day after operation, hypoglycemia and coma suddenly developed, but responded completely after intravenous administration of 50 per cent glucose. During the succeeding days there ensued a series of intense and protracted convulsions. Finally, in spite of continuous administration of intravenous glucose solution and high caloric tube feedings the patient remained in a constant hypoglycemic state and died on April 26, 1957, one month after operation. Blood sugar determinations on numerous occasions were below 30 mg. per 100 ml., even while 10 per cent dextrose solution was being infused

TABLE 1. ROUTINE LABORATORY FINDINGS

Hemoglobin (Gm./100 ml.)	13.0	
Hematocrit	40.2	
White blood cell count (per cu. mm.)	11,650	
neutro-polys (%)	93	
lymphocytes (%)	7	
Sedimentation rate (mm./hr.)	16	
Serum proteins (Gm./100 ml.)	4.5	
albumin/globulin	3.6/0.9	
Serum electrolytes		
CO_2 (mEq./L.)	29.9	
Sodium (mEq./L.)	147	
Chloride (mEq./L.)	103	
Potassium (mEq./L.)	3.9	
Urea nitrogen (BUN) (mg./100 ml.)	9.0	
Glucose tolerance test:	*Blood*	*Urine*
Fasting sugar (mg./100 ml.)	50	1+
½ hour	134	0
1 hour	165	2+
2 hours	210	2+
3 hours	198	0
4 hours	127	0
5 hours	57	0
Insulin in blood (normal 100–300) (micro units/ml.)	1440	
Serum calcium (mEq./L.)	4.6	
Serum phosphorus (mg./100 ml.)	2.4	
Serum alk. phosphatase (Bodansky units)	10.7	
Serum protein-bound iodine (μg./100 ml.)	2.1 and 2.3	

at a rate of 0.4 to 0.65 Gm. of dextrose per minute. Insulin activity in the patient's blood[1] measured 1440 micro units per ml. (normal range, 100–300 micro units using the method of Vallance-Owen *et al.* (10)). The postoperative dextrose intake and blood sugar levels are shown in Figure 1.

During this postoperative period there was rapid development of the full blown physical appearance of Cushing's syndrome with the typical facies, distribution of body weight, striae, and hypertension. The 24-hour urinary 17-ketosteroids rose to 97 mg. and then to 151 mg. prior to stimulation with ACTH. From April 8 to April 12 the patient received ACTH-gel (40 mg. daily) with little effect on urinary corticoids and 17-ketosteroids. The administration of 60 mg. daily of prednisone from April 13 to April 23 produced no amelioration of the hypoglycemia but did effect a significant drop in the total α-ketolic steroid content of the urine from 77 mg. to 12 mg. per twenty-four hours.

Urinary steroids: The urinary steroid values are reported in Table 2. Initial scanning of the chromatograms of the 3-fraction separation presented an unusual steroid pattern. Cortisone, usually found in normal urine, was absent. All of the α-ketolic material found

[1] Assay performed by Dr. Kare Gunderson of the Cox Institute of the Hospital of the University of Pennsylvania.

in the cortisone zone of the toluene-propylene glycol chromatogram consisted of tetrahydro S, in contrast to the normal picture in which as many as 5 α-ketols are seen (11, 12). No aldosterone was detected at the level at which the extract was examined, indicating a value of less than normal. Although large amounts of tetrahydro-B were found, there was no allo tetrahydro-B. At the same time no 3α-allo tetrahydro F was found upon chromatography of the tetrahydro E zone in the chloroform-formamide system as described by Romanoff *et al.* (13) and Bush and Willoughby (14). The predominant metabolite found as the tetrahydro-(ring A reduced) form was of the 3α-normal configuration. This was a departure from the usual pattern in which the 3α-allo forms are always found. The finding of an identifiable amount of corticosterone in the urines of normal subjects to whom corticotropin had been administered has been previously reported. No corticosterone was detected in either of these 2 urines, although the high level of tetrahydro-B indicated the possibility of its presence.

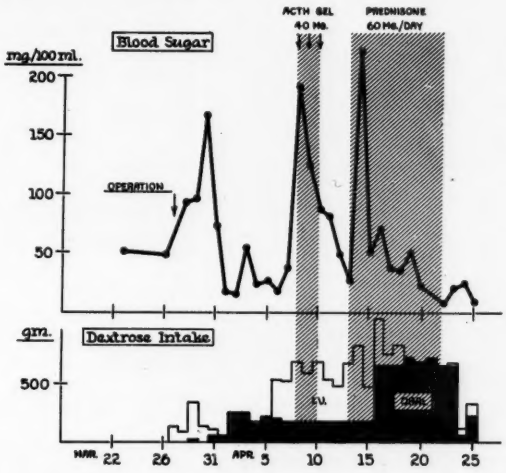


FIG. 1. Marked hypoglycemia in spite of large amounts of dextrose intravenously and orally.

The administration of prednisone caused a decrease in the amounts of the α-ketolic steroids identified. The amounts of tetrahydro-F and tetrahydro-E decreased to the normal range (see Table 2). However, the amounts of F, tetrahydro-S and tetrahydro-B were still elevated, although decreased as a result of administration of prednisone. The levels of tetrahydro-S found in this patient were in the range for patients with metastatic adrenocortical carcinoma previously described (15).

Necropsy findings: The major pathologic changes were found in the pancreas, liver and adrenal glands.

The pancreas was enlarged; it measured 20×6 cm. in greatest diameter, and weighed 200 Gm. It was surrounded by numerous, matted and enlarged lymph nodes measuring up to 3.0 cm. in diameter. In the region of the body of the pancreas there were irregular infiltrating areas of firm gray tumor replacing the normal parenchyma. The involved area was 4 to 5 cm. in length. The pancreatic ducts in this region were thickened and narrowed. The parenchyma in the head and tail was grossly normal. The tumor within

TABLE 2. URINARY STEROIDS IN A CASE OF CUSHING'S SYNDROME WITH HYPERINSULINISM

Compounds*	Normal values (mg./24 hrs.)	Urine I Before ACTH (mg./24 hrs.)	Urine II After ACTH (mg./24 hrs.)	Urine III Before prednisone (mg./24 hrs.)	Urine IV After prednisone (mg./24 hrs.)
17-Ketosteroids	5–15	28,97,151	157		
Corticoids	2–15	123	108		
Total α-ketolic steroids	3–7			77.0	12.0
3α-Normal H_4F	3.2			17.0	3.4
3α-Allo H_4F	1.2			0	0
H_4E	2.0			8.6	2.5
Compound F	0.05			7.8	2.3
H_4S	0.05			11.6	2.5
Aldosterone	0.01			0	0
Compound E	0.05			0	0
3α-Normal H_4B	0.05			4.0	1.0
3α-Allo H_4B	0.05			0	0
Compound B	0.05			0	0
Plasma 17-hydroxy-corticoids	2–10 μg./100 ml.				30 μg./100 ml.

* The compounds were identified by usual procedures and confirmed either by determination of melting point or infrared spectrometry (23, 24).

H_4 = tetrahydro.
F = Hydrocortisone (17-hydroxycorticosterone).
E = Cortisone.
S = 17-Hydroxy-desoxycorticosterone.
B = Corticosterone.

the pancreas was in continuity with retroperitoneal nodes and tumor at the bed of the liver.

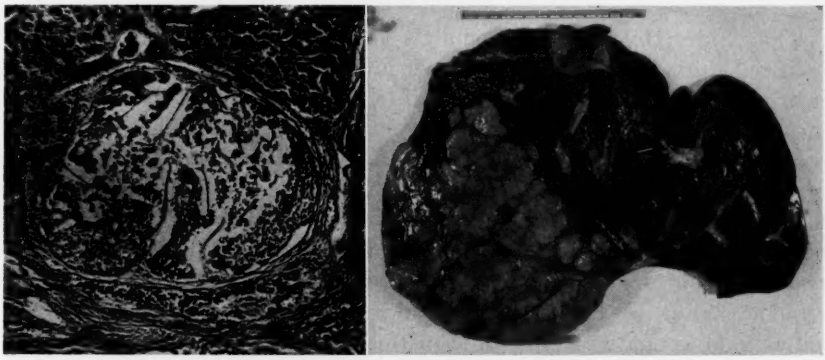
Sections from the head of the pancreas showed increased numbers of distorted, enlarged and irregular islets of Langerhans. Many of these were edematous and markedly vacuolated, with hyperchromatic cells arranged in abnormal architectural pattern. Nests of tumor cells simulating islets were present in adjacent lymphatic channels. The major portion of the glandular parenchyma was undisturbed and essentially normal. The ducts showed no major abnormalities in this portion of the gland.

In the body of the pancreas there was irregular infiltrating carcinoma arranged in broad sheets and cords, and confluent bizarre islands mimicking pancreatic islets (Fig. 2a). The nuclei were round, fairly uniform and hyperchromatic in staining, with pale indistinct cytoplasmic borders. Mitotic figures were rare. In many foci there was a definite pseudoglandular pattern. The tumor infiltrated the acinar portion of the pancreas in an insidious fashion, extending into the fat and into lymphatic channels. Special stains for alpha and beta cells were not successful; the tumor stained an in-

discriminate pale gray without recognizable granularity, in both the primary and metastatic foci.

The liver was markedly enlarged, weighing 4200 Gm. The right lobe was almost totally infiltrated by confluent masses of firm tumor with central necrosis and hemorrhage (Fig. 2b). In the adjacent parenchyma were satellite nodules of tumor. The left lobe of the liver was free of tumor and showed a normal pattern and consistency grossly.

Sections of the right lobe contained a large amount of tumor with an architecture similar to that seen in the pancreas and adjacent lymph nodes. Numerous structures resembling islets were seen. Sections of the left lobe away from the tumor showed normal



a **b**

FIG. 2. **a.** Pancreas with "islets" of carcinoma. **b.** Metastatic tumor in the right lobe of the liver.

portal areas and parenchymal cells. Very scant stainable glycogen was present in the liver cells.

Both adrenal glands were markedly enlarged. Each weighed 21.0 Gm. and measured 9.5×5.0 cm. in greatest diameters. Numerous para-aortic nodes with tumor were attached to the capsule of the right adrenal. On cut section, both glands revealed prominent light tan cortices, 0.8 cm. in thickness, with an exaggerated radiating architecture.

On microscopic examination there was generalized bilateral cortical hyperplasia involving primarily the zona fasiculata and to a lesser degree the zona reticularis. The zona glomerulosa was poorly visualized and compressed beneath the capsule. There was no cortical nodule formation. A single focus of carcinoma was present in the left adrenal cortex which was sharply outlined and had a microscopic appearance similar to other metastatic tumor areas. The medullary regions were preserved and not remarkable.

Additional findings of interest included multiple foci of metastatic tumor in the lungs similar to those described in the pancreas. The lower lobes also contained areas showing severe confluent bronchopneumonia with abscess formation. Extensive metastatic tumor was seen in the lymph nodes from the retroperitoneal, mesenteric, hepatic, pancreatic and paratracheal regions.

No gross abnormality was found in the cranial cavity or the sella turcica. On microscopic examination of the pituitary there was generalized hyperplasia of the anterior lobe with very prominent groups of large pale eosinophilic-staining cells. These cells showed cytoplasmic vacuolization and nuclear ballooning with increased staining, quite

typical of Crooke's hyaline changes of the basophilic cells. No focal circumscribed nodule formation was present. Eosinophilic and chromophobe cells appeared increased in numbers. No abnormality was seen in the pars intermedia or posterior lobe.

Other findings of the postmortem examination, omitted from this report for the sake of brevity, were normal or insignificant.

Pathologic diagnosis: 1) Islet-cell carcinoma of the pancreas with extensive metastases to the liver, lymph nodes and lungs. 2) Marked bilateral adrenal hyperplasia associated with Cushing's syndrome clinically.

DISCUSSION

The clinical features of Cushing's syndrome became strikingly pronounced during the patient's course in the hospital. Consequently the poorly differentiated carcinoma found by biopsy of the liver and adjacent masses was interpreted as adrenal carcinoma. The significance of the preoperative fasting blood sugar level of 50 mg. per 100 ml. and the later onset of severe hypoglycemic episodes was not appreciated until the elevated blood level of insulin-like substance was discovered. Then, with evidence of both adrenal and pancreatic hyperfunction, a final diagnosis was not reached until the necropsy data were available. Reports of multiple endocrine adenomas by Underdahl (5) and Moldawer (6) and their associates led us to investigate parathyroid function and the x-ray appearance of the sella turcica, with negative results. An attempt was made to determine insulin activity post mortem in slices of the primary pancreatic lesion and in slices of metastatic carcinoma. We are unable to explain the absence of assayable amounts of insulin in these tissues.

The lack of response to ACTH administered over a period of three days can be reasonably explained by the assumption that adrenal function was at top capacity prior to additional stimulation. The effect of prednisone over a ten-day period was consistent with results obtained in virilizing adrenal hyperplasia following intravenous hydrocortisone. The total α-ketolic content of the urine dropped from 77 mg. to 12 mg. per twenty-four hours. However, the administered prednisone appeared no more effective than the patient's own excessive corticosteroid excretion in reducing the severity of the hypoglycemia.

It is of interest to speculate on the possible interrelation of adrenal hyperplasia and islet-cell carcinoma of the pancreas with hyperinsulinism. Although the association of these conditions may be coincidental, there are two alternate possibilities.

An islet-cell carcinoma with increased insulin secretion and hypoglycemia could have evoked a pituitary response (Crooke's changes) with secondary adrenal hyperplasia. Then the progressive development of liver metastases and the postoperative depletion of liver glycogen stores may

have accounted for the sudden and intractable hypoglycemia. According to Altszuler *et al.* (16), the anti-insulin effect of 11-17-oxycorticosteroids may be related to their effect on release of glucose from the liver into the blood. Therefore, hypoglycemia in the presence of the damaged liver was not ameliorated by the large amounts of corticosteroids.

The second possibility is the prior existence of adrenal hyperfunction which might have led to pancreatic islet-cell hyperplasia and later neoplasia. This concept receives some support in this particular case from the occurrence chronologically of adrenal symptoms before the symptoms of hypoglycemia. This case would then represent another one of the many malignant lesions found associated with Cushing's syndrome (17–19). With particular reference to the pancreas, there have been acinar carcinoma (1, 2), islet-cell carcinoma without function (3), and now islet-cell carcinoma with hyperinsulinism.

Recent reports by Bush and Willoughby (14) and Romanoff *et al.* (13) indicate that allo-tetrahydro F is a constituent of normal urine. We (11) have shown that allo-tetrahydro B is found in normal male urine. Tetrahydro B and allo-tetrahydro B occur in almost equal proportions. This is true also for these metabolites when isolated from the urine of normal subjects to whom corticotropin had been administered. The absence of both 3-α, allo-tetrahydro F and 3α, allo-tetrahydro B from the urine of the present patient was unexpected. However, Tompkins (20) in his studies with rat-liver enzymes indicated that the predominant enzyme results in formation of the 3α-normal form after reduction of the α,β-unsaturated ketone of ring A of the steroid nucleus.

The finding of excessive amounts of tetrahydro S indicates a failure of the 11-hydroxylating enzyme system to complete the conversion of substance S to hydrocortisone. The production of substance S by the adrenal has been recently shown by its presence in adrenal venous blood (21). It also has been found in large quantities as tetrahydro S in the urine of subjects with adrenocortical carcinoma (22).

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IMPLANTATION OF THE PITUITARY WITH YTTRIUM^{90} IN A CASE OF CUSHING'S SYNDROME

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ABSTRACT

Clinical and biochemical hormonal changes observed in a 27-year-old man with Cushing's syndrome treated by implantation of the pituitary with yttrium^{90} are reported. Eight months after implantation a complete clinical regression of the syndrome was obtained. Hormonal determinations indicated relative adrenal insufficiency, whereas thyroidal and gonadal functions were normal.

CUSHING'S syndrome has been successfully treated by hypophysectomy, electrocoagulation of the pituitary, or section of its stalk (1, 2). Beneficial results have also been obtained through implantation of the pituitary with radon seeds (3, 4) or colloidal Au^{198} (5).

The promising results obtained by us in patients with advanced breast cancer (6) and malignant exophthalmos (7) by implantation of the pituitary with yttrium^{90} induced us to try this treatment in a patient with Cushing's syndrome.

CASE REPORT

A 27-year-old white male presented himself in May 1958 with the clinical picture of Cushing's syndrome. He was obese (height 149 cm., weight 64 Kg.) and had a full-moon face, purple striae, thoracic kyphosis, cervico-dorsal fat pads (Fig. 1a), hypertension and a markedly enlarged heart.

Urinary 17-ketosteroids and 17-hydroxycorticoids were elevated and there was an abnormally pronounced response to the eight-hour intravenous ACTH test (Table 1). The chromatogram[1] of urinary 17-ketosteroids showed an increase in peaks II+III and VI+VII and a marked reduction in peaks IV+V (Fig. 4a).

Osteoporosis was present, both clinically and radiologically. Urinary calcium was moderately increased, but the retention of infused calcium (calcium tolerance test) was abnormally low (Fig. 2). Roentgenologic studies (extraperitoneal pneumography with tomograms, and intravenous pyelography) did not show any abnormality of the adrenals.

On May 28, 1958, one pellet of yttrium^{90} (5.4 mc.) was implanted in the pituitary

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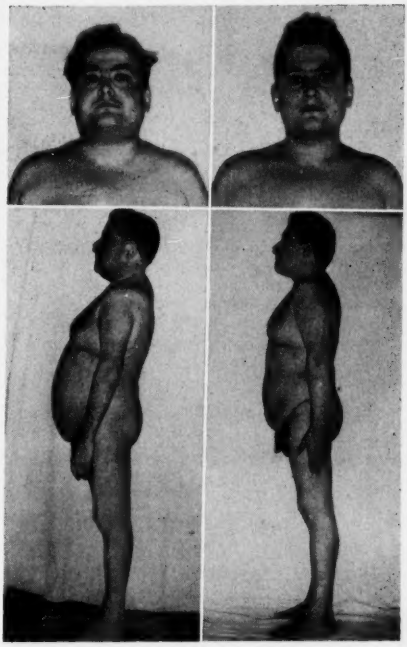
[1] According to Dingemanse *et al.* (8) and Devis (9).

(Fig. 3). Forty-eight hours later there was a sudden drop in blood pressure (80 mm. Hg maximum, 55 mm. minimum) associated with tachycardia, faintness and extreme prostration. Urinary steroids were markedly decreased (17-ketosteroids 2.5 mg., and 17-OH-corticoids 3.4 mg. per twenty-four hours). The state of collapse was rapidly reversed by intravenous administration of hydrocortisone hemisuccinate and noradrenaline. Oral cortisone was started and the dosage was progressively decreased to a maintenance level of 12.5 mg. daily. In the course of the third week the patient exhibited intense desquamation of the skin of the whole body and gradual loss of weight. The 24-hour excretion of 17-OH-corticoids decreased from a normal value of 3.9 mg. on the twentieth post-implantation day, to a very low level thereafter; by the thirty-seventh day none was detectable. The 17-ketosteroids, after a sudden drop immediately following the implantation, remained constantly low (Table 1). Chromatographic analysis of 17-ketosteroids showed a marked decrease in all peaks (Fig. 4b).



a b

FIG. 1. **a)** Before treatment—Cushing's syndrome. **b)** Eight months after implantation of the pituitary with Y^{90}.

Four weeks after implantation, there was a reduction of spontaneous calciuria and to a major extent of induced calciuria, so that approximately 82 per cent of the calcium load was retained (Fig. 2); the serum alkaline phosphatase level was increased.

Three months after the implantation the clinical picture of Cushing's syndrome had disappeared. Blood pressure and heart size were normal. The beard, which had fallen out during the first month, grew normally again. During the fifth month, the third lower right molar tooth erupted. After eight months the patient was in perfect health and working normally (Fig. 1b). He received 12.5 mg. of cortisone daily. However, even when cortisone was discontinued for twenty to thirty days, he did not present the clinical picture of severe adrenal insufficiency. Urinary 17-OH-corticoids were extremely low; the 17-ketosteroids were increased compared with the values obtained one month after implantation (Table 1). Chromatographic analysis of the 17-ketosteroids showed a persistent decrease in peaks II and VI+VII with a relative increase in peaks III and IV (Fig. 4c). The mineral balance was normal. Libido, which had been poor before implantation and absent immediately afterward, had become normal. There were no clinical signs of thyroid insufficiency. The basal metabolic rate and the thyroidal uptake of I^{131} and urinary excretion were normal. The serum level of protein-bound iodine was slightly low and the cholesterol level was moderately increased (Table 1).

TABLE 1. CLINICAL AND HORMONAL DATA BEFORE AND AFTER IMPLANTATION OF YTTRIUM^{90} INTO THE PITUITARY

	Before implantation of 1 pellet of Y^{90} (5.4 mc.)	After implantation	
		1 month	8 months
Body weight (Kg.)	64	59.5	59
Blood pressure (mm. Hg)	160–190/110–130	140–160/80–90	120–130/80–95
Urinary 17-OH-corticoids* (mg./24 hrs.)	28.8–30	0.5–1.3	0.2–0.5
Urinary 17-ketosteroids (mg./24 hrs.)	19–23.4	1.1–1.7	5.5–7
The 8-hour intravenous ACTH test (25 I.U.):			
Urinary 17-OH-corticoids+ (mg./24 hrs.)	47.7	1.9	0.4
Urinary 17-ketosteroids (mg./24 hrs.)	31.4	1.5	4.5
Blood sugar (mg./100 ml.)	120	102	88
Serum cholesterol (mg./100 ml.)	220	240	280
Serum protein-bound iodine (μg./100 ml.)	5.7	—	3.4
BMR (%)	+5	+12	+7
I^{131} test:			
Thyroidal uptake (%): 1 hr.–24 hrs.–48 hrs.	12-40-27	5-36-31	11-35-36
Urinary excretion (%): 0–8 hrs.; 9–24 hrs.; 25–48 hrs.	22-44-5	30-46-3	—

* According to Porter and Silber after enzymatic hydrolysis with β-glucuronidase and sulphatase (10).

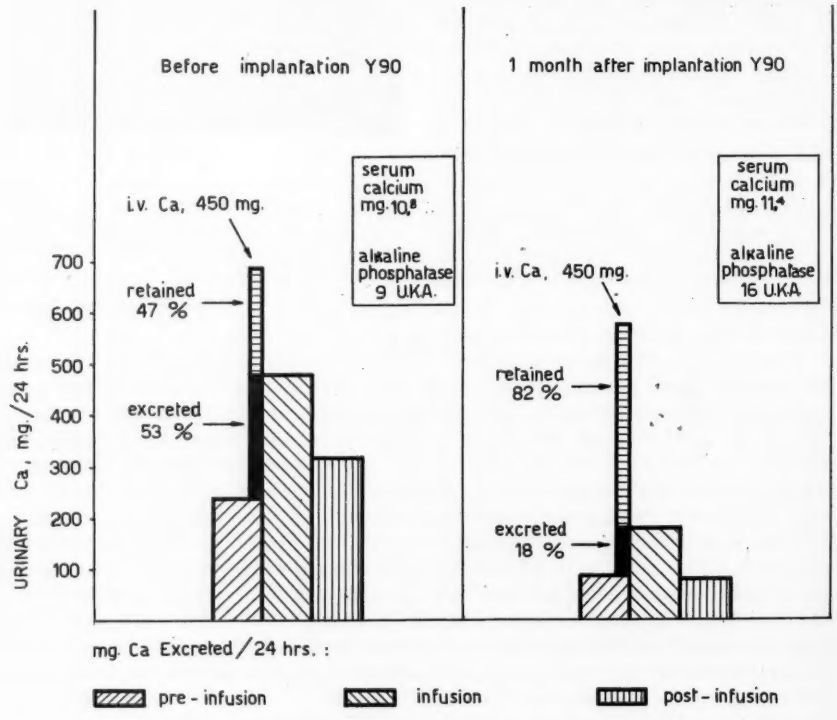


FIG. 2. Calcium tolerance tests performed before and after implantation of the pituitary with Y^{90}.

FIG. 3. Roentgenogram showing front and side views of the skull after implantation of Y^{90} into the pituitary.

DISCUSSION

In this patient, implantation of the pituitary with yttrium^{90} induced a complete regression of Cushing's syndrome. The drop in the excretion of urinary steroids and the cardiovascular collapse immediately after the implantation suggest that yttrium^{90} had caused a sudden suppression of ACTH secretion.

The production of ACTH seemed to undergo further inhibition after the twentieth day, as indicated by the gradual decrease in urinary 17-OH-corticoids after this date. An alternative explanation is that exhaustion of the hypertrophic adrenals followed the total suppression of ACTH only after a certain lapse of time.

A single dose of ACTH (the eight-hour intravenous ACTH test) appeared insufficient to induce an increase of urinary steroids. The effect of more prolonged dosage in increasing steroid excretion was determined by daily administration of 20 I.U. of ACTH-gel (Richter) for five days (Fig. 5). The lack of response to a single dose of ACTH eight months after the implantation demonstrates the profound functional quiescence of the adrenal cortex. The progressive increase of the activity of this gland following repeated administration of ACTH-gel confirmed the hypothesis that the hypofunction was due to insufficient stimulation by the pituitary.

It is noteworthy that when the substitutive administration of cortisone was discontinued in order to perform hormone determinations, no sign of melanoderma was observed. The eruption of a molar tooth, the normal

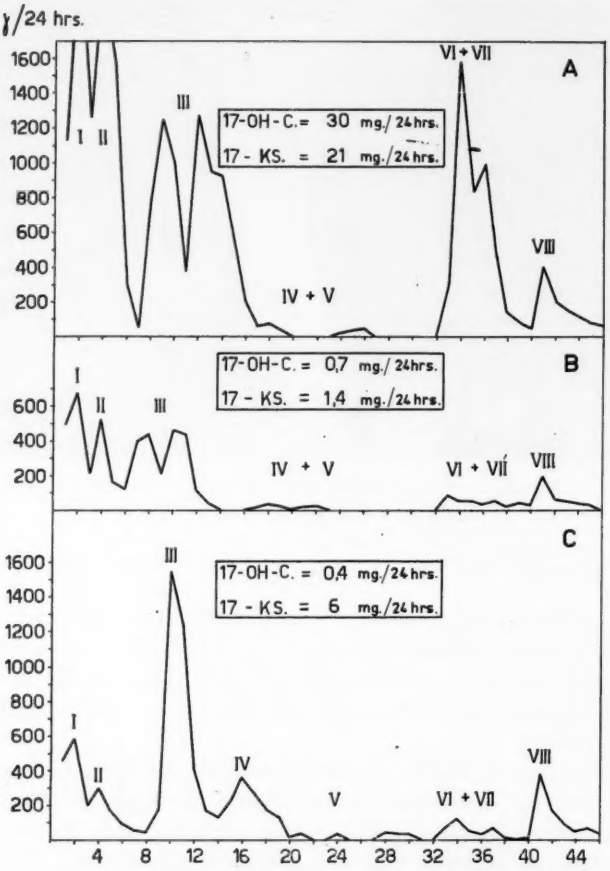


FIG. 4. Chromatography of urinary 17-ketosteroids: **A**) Before implantation of Y^{90}—increase in peaks II, III and VI+VII; marked decrease in peaks IV+V. **B**) One month after implantation—marked reduction in all the chromatographic peaks. **C**) Eight months after implantation—the decrease in peaks II and VI+VII is still evident; a relative increase in peaks III+IV is observed.

growth of the beard, the presence of normal libido and the absence of signs of hypothyroidism permit the assertion that other functions of the pituitary were adequate eight months after implantation with yttrium^{90}.

The consistent decrease in the chromatographic peaks VI and VII confirms the insufficient incretion of glycocorticoids, and the relative increase in peaks III and IV associated with the increase of total 17-ketosteroids testifies to the recommencement of the secretion of androgens.

Similar observations have been reported by Northfield (4) in a case of Cushing's disease treated by radon implantation of the pituitary, and by Arner and associates (1) in another case after electrocoagulation of the gland. In both instances, clinical remission was accompanied by an almost normal function of all the other ductless glands.

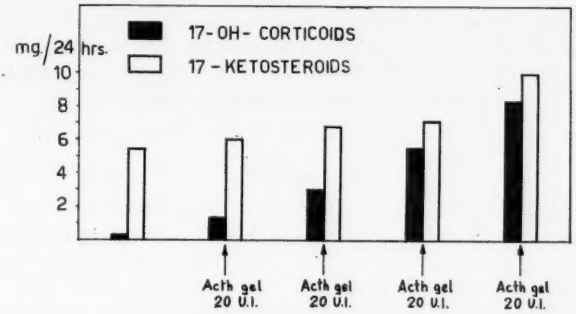


FIG. 5. Progressive increase in urinary steroids after the administration of four daily doses of 20 I.U. of ACTH gel.

That it is possible by means of yttrium^{90} implantation to correct the hypersecretion of one hormone of the pituitary without inducing total hypopituitarism has been previously reported by us in 2 cases of malignant exophthalmos (7). The present observation lends support to the previously presented hypothesis that implantation of the pituitary with yttrium^{90} causes a functional inhibition of only those cells which are pathologically hyperactive, without injury to the normal tissue (7). This could be explained on the basis of the fundamental rule of radiobiology that hyperactive cells are much more sensitive to irradiation than are normal cells.

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JCEM

THE DETERMINATION OF ALDOSTERONE IN URINE*†

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ABSTRACT

A procedure for determination of aldosterone in a 24-hour sample of urine is described. The aldosterone conjugate in urine is hydrolyzed with acid, the free steroids are extracted with chloroform, and the aldosterone is separated from the other steroids by successive chromatograms in 1) formamide-chloroform, 2) formamide-butyl acetate-water, and 3) ethyl acetate-toluene-benzene-water. The quantity of aldosterone present is estimated from the intensity of both the blue spots and the yellow fluorescent spots that are obtained by appropriate treatment of the final chromatogram with alkaline blue tetrazolium.

A determination requires three days. Normal values range from 2 to 16 μg. per twenty-four hours, and recoveries of 5-μg. to 10-μg. quantities are 80 to 90 per cent. Dilute acetic acid acts as a preservative for samples of urine that cannot be refrigerated. Various factors that affect the determination are discussed.

SEVERAL procedures for the determination of urinary aldosterone have been described. Some appear to lack the desired specificity for aldosterone; others are rather long and tedious and not well suited for routine use. The following procedure requires three days for completion, utilizes relatively simple apparatus and technics, and is generally satisfactory for determination of normal or elevated amounts of aldosterone in a 24-hour sample of urine in a clinical laboratory. It is based on the aldosterone method of Neher and Wettstein (1).

MATERIALS

All solvents employed are of good quality and are redistilled. Formamide is redistilled in vacuo; but some samples of it, which are colorless and odorless when received, are

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used without being redistilled. Methylene chloride and chloroform are distilled over anhydrous Na_2CO_3.

The short-wavelength ultraviolet lamp is a quartz spiral resonance mercury lamp, Model C-2371, of the Hanovia Chemical and Manufacturing Co., Newark, New Jersey. It is attached to the secondary winding of a transformer that is rated at 3000 volts and 120 milliamperes when its primary winding is connected to a 110-volt power line. In our application, a variable transformer is inserted in series with the primary winding to reduce the primary voltage to 70 volts. The lamp is enclosed in a wooden box 2 feet square and 8 inches deep. A hole 2.75 inches in diameter in the center of the top is fitted with a 3 by 3-inch Corning 9863 filter that absorbs the visible light and transmits the 254-mμ line. The lamp is mounted in the box 1.33 inches from the outer surface. With this apparatus small amounts of 3-keto-Δ^4-steroids can be detected; the 1-μg. spot of cortisol which has migrated 10 cm. in the final chromatogram described subsequently is visible. Suitable glasses are worn to protect the eyes during this procedure. (If the lamp is to be used longer than five minutes at a time, it should be cooled with a current of air.)

The long-wavelength ultraviolet lamp consists of two G.E. F8 T5-BLB fluorescent lamps mounted in a Lite-Mite DL-28 desk light, from Stocker and Yale, Marblehead, Massachusetts.

Blue tetrazolium [3,3′-dianisole-bis-4,4′-(3,5-diphenyl)-tetrazolium chloride] from Synthetical Laboratories, Chicago, Illinois, is recrystallized from alcohol-ether (2).

The chromatography paper is Whatman No. 1—Special for Chromatography, and Schleicher and Schüll 2043A.

Cortisol, Mann Research Laboratories, New York, N. Y., is recrystallized from methanol (melting point, 216° to 219° C.). Standard solutions that contain 4.0, 3.0, 2.0, 1.0 and 0.5 μg. per 10 μl. of methanol are prepared. Solutions prepared in specially cleaned glassware or in ordinary glassware with 10 μg. of disodium ethylenediaminetetraacetic acid per ml. do not undergo detectable deterioration when kept as long as two months at 4° C. Some solutions, stored in ordinary glassware known to contain traces of copper ion (3), undergo noticeable deterioration in less than two weeks.

PROCEDURE

Collection and storage. A 24-hour sample of urine is collected in a glass bottle. If the specimen can be kept cold, no preservative is added. If it cannot be refrigerated, the specimen is collected in a bottle that contains 15 ml. of acetic acid (1 volume of glacial acetic acid plus 2 volumes of water) as a preservative. For hydrolysis of the conjugate, the pH is adjusted to 1.0 with concentrated hydrochloric acid and the sample is allowed to stand at room temperature for eighteen hours.

Extraction. The urine is extracted in a separatory funnel with 60 per cent of its volume of chloroform divided into four equal portions. The combined chloroform extract is washed with 15 per cent of its volume of a sodium hydroxide-sodium carbonate solution (0.1 *N* sodium hydroxide and 5 per cent sodium carbonate) and then with 15 per cent of its volume of distilled water until the aqueous wash is neutral to litmus. Each aqueous wash is extracted with a small volume of chloroform and the organic phase is added to the main chloroform extract before proceeding to the next wash. The chloroform extract is evaporated to dryness in vacuo in an all-glass still in a water bath that is kept below 50° C. The residue is transferred to a small tared test tube with methanol and the solution is taken to dryness in a stream of carbon dioxide. The weight of the residue is determined.

First chromatogram (*Fig. 1a*). Sheets of unwashed Whatman No. 1 paper, 18 by 52 cm., are drawn through a mixture of 30 per cent formamide in acetone and hung on a glass rod with stainless steel clips. The residue from the urinary extract is dissolved in 100 μl. of 1:1 chloroform-methanol, the test tube is stoppered and centrifuged, and the solution is applied along a 13-cm. line on two or more papers. Usually a 24-hour extract will weigh less than 40 mg. and can be applied to two sheets of paper. If the weight is greater than 40 mg., more than two sheets of paper are required. The test tube is rinsed with two successive 25-μl. portions of 1:1 chloroform-methanol, and the rinsings are applied to the chromatogram. As a control, 20 μg. of cortisone in 10 μl. of methanol is applied.

By the general technic previously described (4), the chromatogram is developed in chloroform saturated with formamide until the solvent front is near the bottom of the paper (about two hours). The paper is removed from the jar, and after a few minutes

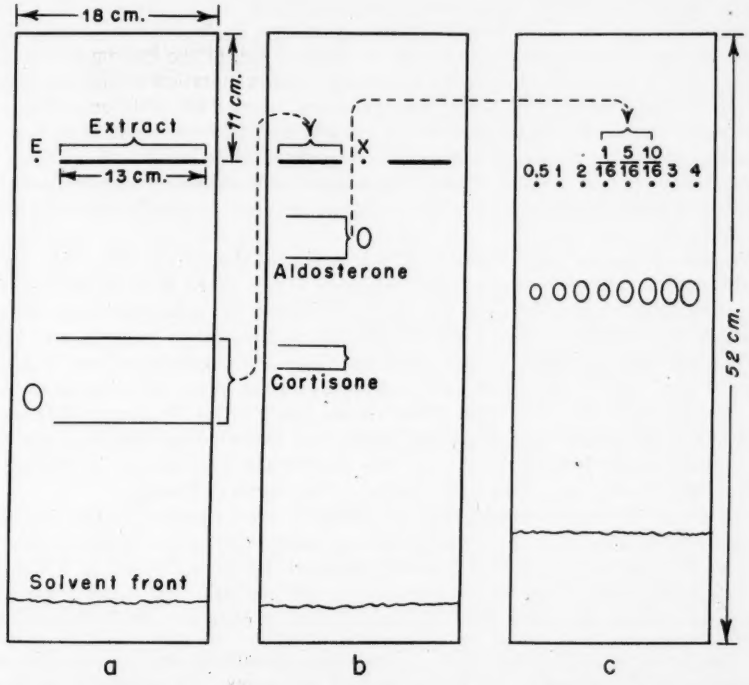

FIG. 1. a. Formamide-chloroform system separates aldosterone and cortisone from most of the other steroids, and removes about 90 per cent of the total solids. "E" designates point of application of cortisone. b. Formamide-buytl acetate-water system separates aldosterone from cortisone and several other substances, and reduces the weight of the aldosterone fraction to 0.1–0.4 mg. "X" designates point of application of the aldosterone control or of 9α-hydroxycortisol. c. Toluene-ethyl acetate-methanol-water system removes interfering substances frequently not removed by previously applied systems.

it is viewed briefly over 254-mμ illumination and the zones of interest are marked. A zone from each paper extending 1 cm. below and 6 cm. above the bottom of the cortisone[1] standard is cut out and placed in a glass-stoppered Erlenmeyer flask. About 10 ml. of water per 100 sq. cm. of paper is added and the flask is shaken vigorously until the paper disintegrates. Then after it has set for fifteen minutes, the solution is filtered from the pulp. Suction is used and the pulp is pressed down onto the funnel with a glass rod to aid in removing the solution. The pulp is washed twice with 5-ml. portions of water. The aqueous eluate is extracted with three 10-ml. portions of chloroform, and the combined extract is washed with 3 ml. of water and evaporated to dryness in vacuo. The residue is transferred to a small test tube with methanol and the solvent is removed in a stream of carbon dioxide. The residue usually weighs 1 to 4 mg.

Second chromatogram (*Fig. 1b*). A paper is drawn through 30 per cent formamide in acetone and the steroid from the previous chromatogram is applied along a 6-cm. line with 25 μl. of 1:1 chloroform-methanol and two 10-μl. rinses. For a standard, 5 to 10 μg. of aldosterone or 5 to 10 μg. of 9α-hydroxycortisol[2,3] (which moves 0.93 as fast as aldosterone) is applied. The chromatogram is developed in the formamide-water-butyl acetate system (4) until the solvent front is within 5 cm. of the bottom of the paper (about three to three and a half hours required). After evaporation of the mobile phase the standard is located over 254-mμ illumination and a zone 8 cm. wide, extending 1 cm. above and 1 cm. below the standard spot (1 cm. above and 1.5 cm. below if 9α-hydroxycortisol is used as the standard) is removed. This zone is eluted with 5 ml. of water in a small Erlenmeyer flask and the mixture is processed as described for the first chromatogram except that correspondingly smaller volumes are used. This residue usually weighs 0.1 to 0.4 mg.

Final chromatogram and estimation. The intensity of the 254-mμ absorbing zone in the aldosterone area of the second chromatogram can be taken as an indication of the amount of aldosterone present. If the residue is judged to contain not over 30 μg. of aldosterone[4] it is dissolved in 1.6 ml. of methanol and portions of 0.1, 0.5 and 1.0 ml. are removed and taken to dryness in small test tubes in a stream of carbon dioxide. By means of 20 μl. of 1:1 chloroform-methanol and two 10-μl. rinses the aliquots are transferred to Whatman No. 1 (or S&S 2043A) paper (see Fig. 1c). Standards that contain 0.5, 1.0, 2.0, 3.0, and 4.0 μg. of cortisol in 10 μl. of methanol are placed on the paper 2 cm. apart at the locations indicated. The papergram is developed in system C of Bush at 25° C. after an equilibration period of two hours or longer.

The lowest 12 cm. is removed from the finished, dried chromatograms. An area of the paper from about 3 cm. above the origin to about 8 cm. below the aldosterone area is drawn through a solution that is freshly prepared by mixing 90 ml. of 2 *N* sodium hydroxide and 10 ml. of blue tetrazolium (0.1 per cent in water). The paper is then placed on a large glass plate over a white background and a glass rod about 26 cm. long is

[1] The chromatographic mobilities in formamide-chloroform and in formamide-butyl acetate-water of aldosterone, cortisone, cortisol and several other steroids which may be present in the crude urinary extracts used for aldosterone assay have been reported previously (4).

[2] We wish to thank Dr. Karl Pfister of Merck & Co., Inc., Rahway, N. J., for a supply of 9α-hydroxycortisol.

[3] Triamcinolone migrates in this solvent system at virtually the same rate as aldosterone; 16α-hydroxycortisol and 2α-hydroxycortisol migrate at 1.1 and 0.92, the rate of aldosterone.

[4] If it contains more than 30 μg., an appropriate aliquot is used.

placed under the paper about 1 cm. above the origin. The head of the chromatogram is torn off 2 cm. above the origin and the tail is removed 4 cm. below the aldosterone areas. The immediate removal of these dry portions of the chromatogram is essential if the blue spots and the subsequently produced soda-fluorescent spots are to coincide. The intensity of the blue spots in the appropriate aliquots is read by 2 observers against the standards after three to five minutes.

The paper is attached to the rod on which it is lying, transferred to a glass rack (Fig. 2) and dried without delay at 85–100° C. in an oven not equipped with forced draft. Then it is evaluated under long-wavelength ultraviolet illumination. Usually the intensities of the blue spots and the yellow fluorescent spots are within less than 20 per cent of each other. An average of the two values is taken and multiplied by the appropriate factor to obtain the amount of aldosterone present. For example, if the material was divided into portions of 0.1, 0.5, and 1.0 ml. and if the average value for the 1.0-ml. aliquot was 2.5, the total quantity of aldosterone in the sample would be

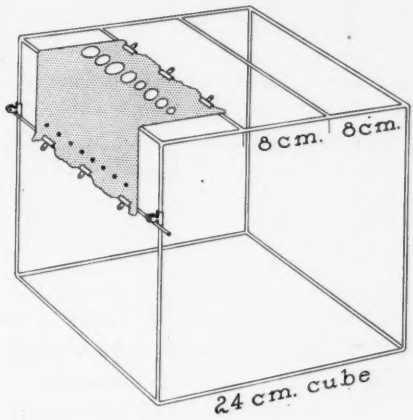

FIG. 2. Glass frame (made from 6-mm. rod) prevents paper from curling when it is dried. Two papers may be dried simultaneously on the frame.

$$2.5 \times \frac{1.6}{1.0} = 4\ \mu g.$$

RESULTS

In 34 healthy persons (19 men and 15 women, from 22 to 63 years old) whose salt intake was unrestricted, the excretion of aldosterone ranged from 2 to 16 μg. per twenty-four hours, with 5.6 μg. as the average. There was no significant difference in the values between the men and the women. The average weight of the crude urinary extract was 25 mg., with extremes of 15 and 46 mg. Urinary extracts obtained with either methylene chloride or chloroform contained approximately the same amounts of total solids.

Fourteen replicate determinations were run on a large pool[5] of extract.

[5] In the routine determination of formaldehydogenic steroids in our laboratory, the hydrolysis of conjugates at pH 1 and extraction of urine is performed exactly as in the

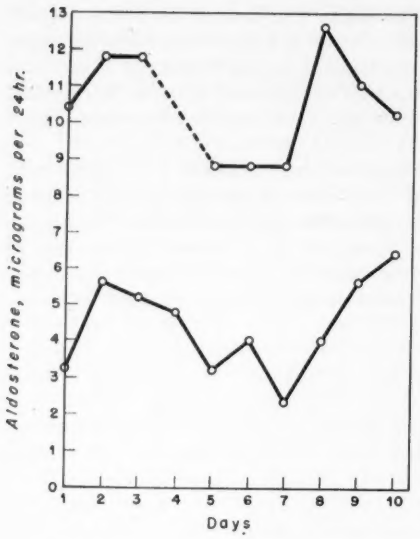

FIG. 3. Aldosterone values from urine of 2 normal men on ten consecutive days.

The quantity of aldosterone found was 5.1 ±0.45 μg. (S.D.) per twenty-four hours, and the range was from 4.8 to 6.4 μg.

The values from the urine of 2 normal men for ten consecutive days are given in Figure 3.

Frequently it is not convenient to store urinary samples at reduced temperatures or to analyze them immediately after they are collected. In order to study the stability of aldosterone in urine that had stood several days at room temperature, the following experiments were performed. A pool of fresh urine from both males and females (4500 ml.) was divided into three 1500-ml. aliquots. One aliquot was adjusted to pH 1 and processed in the usual manner without delay. A second aliquot was mixed with 15 ml. of acetic acid (5 ml. glacial acetic acid plus 10 ml. water) and allowed to stand for four days at room temperature (25 to 28° C.) before adjustment to pH 1 and extraction. A third aliquot, to which no acetic acid was added, was used as a control for the second. Determinations were performed on aliquots from 5 different pools of urine (Table 1). In experiments with 2 pools no significant difference was found among the amounts of aldosterone measured in the 3 kinds of aliquots. In the other 3 pools all of the aldosterone was destroyed in the aliquots without the acetic acid, but the amounts of aldosterone measured in the samples containing acetic acid and in the samples processed immediately were not significantly different from each other.

Nowaczynski and associates (5) reported that slightly greater yields of aldosterone were obtained by continuous chloroform extraction of urine at pH 1 in a Cohen-type extractor than were obtained by noncontinuous extraction. Walraven (quoted by Ayres and associates (6)) obtained two to three times as much aldosterone by use of continuous extraction as by the normal procedure.

Table 2 shows results of our determinations in which hydrolysis of aldo-

above procedure for aldosterone. This pool represents an aliquot from each of more than 100 persons whose corticosteroid levels were determined in the course of clinical diagnosis. Samples of this extract have been stored in methanol in stoppered test tubes at 4° C. for longer than six months without loss of aldosterone.

TABLE 1. PRESERVATIVE EFFECT OF ACETIC ACID ON ALDOSTERONE IN URINE

Pool No.	Aldosterone (μg. per 1500-ml. aliquot)		
	Fresh	After 4 days, HOAc added	After 4 days, no HOAc added
1	5.6	5.2	4.7
2	4.8	4.8	4.8
3	5.7	5.6	0
4	5.6	4.4	0
5	3.7	3.9	0
Mean	5.1	4.8	—

sterone was effected 1) in the routine manner, 2) by continuous extraction with 225 ml. of chloroform in a Cohen (7) extractor, and 3) by continuous mechanical stirring in the presence of 225 ml. of chloroform. The latter two procedures yielded intractable emulsions that had to be filtered one or more times before the phases could be separated, and did not produce significantly greater yields of aldosterone than did the routine procedure.

In further studies a 5-liter round-bottomed flask containing a 1500-ml. aliquot of urine at pH 1 and 225 ml. of chloroform was placed on a shaking

TABLE 2. EFFECTIVENESS OF METHODS FOR EXTRACTING ALDOSTERONE FROM 1500-ML. ALIQUOTS OF POOLS OF URINE

Pool No.	Micrograms of aldosterone		
	Routine procedure*	Continuous extraction†	Stirring‡
1	5.0	5.8	5.3
2	6.1	7.3	7.6
3	4.8	5.0	5.3
4	8.1	7.9	8.6
5	6.8	6.5	6.8
6	5.6	7.4	5.5
Mean	6.1	6.6	6.5

* Urine remained at pH 1 for 18 hours, then was extracted by four 225-ml. portions of chloroform.

† Urine was extracted continuously for 18 hours at pH 1 with 225 ml. of chloroform in a Cohen extractor, then extracted in a separatory funnel with three 225-ml. portions of chloroform.

‡ Urine was stirred continuously for 18 hours at pH 1 with 225 ml. of chloroform, then extracted in a separatory funnel with three 225-ml. portions of chloroform.

machine that provided gentle agitation (not enough to cause emulsification) for eighteen hours. Subsequent extraction with additional portions of chloroform and chromatographic analysis gave the results shown in Table 3. It is evident that this hydrolytic procedure produced considerably more chloroform-extractable material than did the routine procedure, but did not increase appreciably the yield of aldosterone.

TABLE 3. WEIGHTS OF RESIDUE AND QUANTITIES OF ALDOSTERONE OBTAINED WITH ROUTINE PROCEDURE AND WITH CONTINUOUS EXTRACTION

Pool No.	Routine procedure		Continuous extraction	
	Total wt. (mg.)	Aldosterone (μg.)	Total wt. (mg.)	Aldosterone (μg.)
1	34	8.0	74	8.0
2	38	5.6	63	6.4
3	26	5.1	36	6.5
4	38	5.6	29	5.6
5	67	3.7	105	3.3
Mean	41	5.6	61	6.0

Recoveries of 5.0-μg. and 10.0-μg. quantities of aldosterone added to 1100-ml. aliquots of fresh male urine, which assayed 2.6 μg. of aldosterone, were 89 and 98 per cent, respectively.

Duplicate 1000-ml. aliquots from a pool of fresh male urine yielded 4.2 μg. and 4.4 μg. of aldosterone. Recoveries of 5.2-μg. amounts were 79 and 87 per cent and recoveries of 10.4-μg. quantities were 82 and 93 per cent.

Recoveries of 1-μg. amounts added to samples of extracts (and therefore not submitted to the hydrolytic procedure) which contained 0.0 μg. and 0.3 μg. were 75 and 90 per cent.

In chromatographic systems that include formamide as a component, frequently ammonia forms and by its presence renders alkaline the atmosphere in the chromatography jar. No loss of aldosterone was observed when it was chromatographed in systems of formamide-chloroform or formamide-butyl acetate-water in jars in which a beaker that contained 1 ml. of concentrated ammonium hydroxide was placed.

It has been found that the presence of traces of cupric ion in the glassware used in the chromatographic purification of steroids causes serious loss of cortisone and cortisol and minor loss of aldosterone (3). Most of the destruction appears to occur while the steroids stand in test tubes in methanol or in the dry state. Destruction also occurs during equilibration

of chromatograms by systems of the Bush type, but relatively little destruction occurs during chromatography by Zaffaroni-type systems. The presence of disodium ethylenediaminetetraacetic acid (EDTA) protects these steroids against copper. Good recoveries of cortisol and cortisone are obtained when they are transferred to paper with methanol that contains EDTA, the chromatogram is run in the routine manner, the steroids are eluted with water, and the eluate is extracted from water with chloroform to which EDTA in methanol is added prior to removal of the solvent.

When this technic is followed in the determination of aldosterone in pools of urine, the addition of 100 μg. of EDTA to the chloroform extract at each stage of the procedure does not increase significantly the amount of aldosterone found. Its presence does prevent the formation of a trace of soda fluorescence at the origin of the final chromatogram, but the quantity of this fluorescence (equivalent to about 0.1 μg. of aldosterone) is small in comparison with that of the unaltered aldosterone (about 4 μg.).

The elution of aldosterone from paper with water by making a pulp, filtering, and washing with water is rapid and efficient. Subsequent extraction of the pulp with methanol gave less than 1 per cent of additional cortisone or aldosterone.

Aldosterone in strips of paper has been eluted with water chromatographically also. This has been done by cutting a broad "V" into one end of the paper, attaching the opposite end to a wet wick composed of a double thickness of Whatman No. 1 paper set up for descending flow, and collecting 1 ml. per 10 sq. cm. of paper.

Various weights (11 mg., 22 mg., 33 mg., and 44 mg.) of residue from a pool of extract have been chromatographed on a 13-cm. line on Whatman No. 1 paper impregnated with 30 per cent formamide in acetone. Chromatograms were overloaded with 44 mg. of residue; with 33 mg. the zones were considerably wider than with 22 mg., and with 22 mg. they were slightly wider than with 11 mg.

Superior initial resolution of the steroids in the extract may be achieved by the following maneuver. The first chromatogram is developed in benzene saturated with formamide until the front is near the bottom of the paper. It is then removed, dried for five minutes, and developed in chloroform in the usual manner. With this technic, which requires an additional three hours, the bands containing cortisol, cortisone, and so on are made much narrower than when the benzene development is omitted. The heavy ultraviolet-absorbing zone, which ordinarily moves at approximately the same rate as cortisone, moves significantly faster and is nicely separated from cortisone and aldosterone. However, use of this procedure does not ordinarily improve the over-all result achieved in the final chromatogram.

Occasionally urinary extracts contain substances that are highly fluores-

cent when viewed over 254-mμ illumination. These substances may mask the dark zones ordinarily observed from 3-keto-Δ^4 steroids. Usually a Willemite screen absorbs the fluorescence from these materials in the extract and the 3-keto-Δ^4 steroids are revealed as dark areas.

COMMENT

The foregoing procedure for quantitation of aldosterone is based on the original procedure of Neher and Wettstein (1). Certain parts of their procedure have been simplified. The drying and photographing of the first chromatogram for location of the ultraviolet-absorbing steroids have been eliminated. Areas containing these steroids are detected by viewing the wet chromatograms briefly over 254-mμ illumination. Areas of interest are marked with a pencil. The steroids are eluted from the papergrams without removal of the nonvolatile stationary phase.

An additional chromatogram (formamide-butyl acetate-water) has been introduced to purify further the crude aldosterone before it is quantitated. Without this additional papergram, frequently the spots obtained from the aldosterone aliquots with alkaline blue tetrazolium were of a slightly different color and much larger than those formed from the cortisol standards. Under these circumstances valid quantitation could not be made. This chromatogram separates aldosterone from 5 or 6 other compounds that also absorb illumination at 254 mμ, produce color with alkali, reduce blue tetrazolium, or produce fluorescence with alkali.

The Neher-Wettstein method of quantitation of aldosterone has been retained: the intensities of both the blue spots and the yellow fluorescent spots, obtained from alkaline blue tetrazolium, are compared visually with those of the standards. Although such a method is subject to ± 20 per cent error in estimation, it has the advantage of being relatively simple and of using both the α-ketolic side-chain and the α,β-unsaturated ketone in the quantitation, which add to its specificity. When the blue and the fluorescent spots coincide in location, area and intensity, one feels assured of measuring aldosterone. If they do not agree, sufficient purification of the aldosterone has not been achieved.

Unwashed paper has given results as good as paper washed thoroughly with 95 per cent alcohol and rinsed with benzene. Although Whatman No. 1 paper has been employed in most of the experiments, S&S 2043A is preferable for the final chromatogram because it has significantly greater wet-strength than Whatman No. 1. Handling of the final chromatogram is made easier by use of a single sheet of paper rather than a head with legs attached (1).

Under the conditions used for evaluating the quantities of steroids on the final chromatogram, aldosterone reacts with alkaline blue tetrazolium

almost instantaneously to form a blue color but cortisol reacts somewhat more slowly. The optimal time for comparison of the spots from the unknown with standards is three to four minutes after treatment with tetrazolium; subsequently the color appears to fade slowly as the background from the paper increases.

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A SIMPLIFIED METHOD FOR THE SIMULTANEOUS DETERMINATION OF 17-KETOSTEROIDS, DEHYDROEPIANDROSTERONE, AND 17-HYDROXYCORTICOSTEROIDS IN SERUM

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ABSTRACT

A simple procedure is described which permits the determination for clinical purposes of 17-hydroxycorticosteroids, 17-ketosteroids and dehydroepiandrosterone in only 10 ml. of serum. The process of extraction and purification of the hydroxycorticosteroids follows the procedure of Nelson-Samuels coupled with the Silber-Porter assay. The method for determination of the 17-ketosteroids incorporates the principles of solvolysis as described by Burstein and Lieberman. This procedure yields results equivalent to those obtained with the more tedious alcohol extraction followed by simultaneous hydrolysis and extraction with ether at pH 1. A clean-up procedure based on the Drekter sodium hydroxide pellet technique has been used and found to aid in improving the specificity of the method. A micro-technique has been developed for the blue chromogen reaction of Allen and applied to the determination of dehydroepiandrosterone. A discussion of the development of the method is included, together with values for a group of normal males and females.

MANY investigators (1–10) have described procedures for the determination of 17-ketosteroids in serum. The early methods (1–3) seemed to lack the desired specificity, and the others (6–9) either have not been adapted to routine methods or have been specific for only one of the 17-ketosteroids. Gardner (4), Migeon and Plager (5), and more recently Ceresa and Cravetto (10) have published methods requiring smaller quantities of serum. The procedure to be described permits the determination for clinical purposes of free 17-hydroxycorticosteroids, of 17-ketosteroids as measured by the Zimmermann (1) reaction, and dehydroepiandrosterone as measured by the Allen (11) reaction.

In this method, the need for continuous hydrolysis and extraction has been eliminated by applying the principles of solvolysis described by Burstein and Lieberman (12) in their recent publication. A clean-up procedure instituted prior to column chromatography has helped to increase the

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specificity of the method, and a micro-technique has been applied to the Allen reaction which permits the simultaneous simple determination of dehydroepiandrosterone. In the course of the investigative work which led to the final development of the present method, some observations were made which may contribute to a better understanding of the complexities of such procedures.

PROCEDURE

Collection of Serum

Collect 25–30 ml. of whole blood, allowing it to stand no longer than necessary. Loosen the clot, centrifuge the specimen and pour off the serum. Centrifuge, decant and re-centrifuge. Pour into a 50-ml. Erlenmeyer flask. Freeze within one hour after collection and start tests within one week.

A. *Separation of Serum 17-Hydroxycorticosteroids*

Extract 10 ml. of serum three times with 15 ml. of redistilled chloroform as described by Nelson and Samuels (13, 14) for the determination of corticoids. Combine the chloroform extracts, evaporate, then chromatograph according to the Nelson-Samuels technique and assay by the Silber-Porter (15) procedure. The aqueous portion contains practically all of the serum 17-ketosteroids.

B. *Extraction, Chromatography and Solvent Partition of Serum 17-Ketosteroids*

1. *Ethyl-acetate extraction*
   a. Drain the aqueous portion obtained in Part A from the separatory funnel into a graduate.
   b. Rinse the funnel with water and add the rinsings to the graduate to make the volume up to 16 ml.
   c. Transfer to a 125-ml. Erlenmeyer flask and add an additional 16 ml. of water. Use this water to rinse the graduate. The total volume is now approximately 32 ml.
   d. Adjust pH to 1.0 with a few drops of 18*N* H_2SO_4.
   e. Add 3.6 ml. of 18*N* H_2SO_4 to make a 2*N* solution.
   f. Add 40 ml. of ethyl acetate to the acidified emulsion, swirl, and mix well.
   g. Transfer to 50-ml. round-bottom centrifuge tubes, cap with foil and centrifuge ten minutes.
   h. Decant the clear ethyl-acetate extract into a clean 125-ml. Erlenmeyer flask.
   i. Wash the precipitated material four times with 10-ml. portions of ethyl acetate.
   j. Centrifuge for five to ten minutes after each wash and combine the ethyl-acetate extracts.

2. *Hydrolysis (solvolysis in ethyl acetate)*

Incubate the combined ethyl-acetate extracts from Part 1 at 37° for twenty-four hours.

3. *Removal of phenolic, acidic and pigmented material*
   a. Wash the ethyl-acetate extract from Part 2 once with an equal volume of 1 per cent $NaHCO_3$.
   b. Filter into an Erlenmeyer flask and evaporate to dryness with an impinging air stream.

c. Add 5 ml. of repurified redistilled ethylene chloride to the dry residue. Rinse flask twice with 2.5 ml. of ethylene chloride to make a total volume of 10 ml. of ethylene chloride.
d. Transfer to a 50-ml. stoppered centrifuge tube.
e. Add approximately 20 pellets of NaOH, stopper, and shake fifteen minutes in a shaker (Drekter *et al.* (16)).

4. *Column chromatography*
a. Prepare a column (10 mm. in diameter) with 3 Gm. of Florisil. Tap Florisil into column.
b. Wash column with 10 ml. of ethylene chloride and discard.
c. Filter the ethylene-chloride extract from Part 3 through a #1 Whatman filter paper directly onto the column. Rinse the centrifuge tube with 2.5 ml. of ethylene chloride and add to the column.
d. Elute with 35 ml. of 2 per cent methanol in redistilled $CHCl_3$ and collect the entire 47 ml. into an Evelyn colorimeter tube or flask.
e. Evaporate to dryness with an impinging air stream.

5. *Solvent partition*
a. Take up residue from Part 4 in 3 ml. of 70 per cent ethanol.
b. Add 3 ml. of hexane and shake 15 seconds.
c. Centrifuge and remove hexane with suction.
d. Repeat (b) and (c) twice.
e. Add 3 ml. of water to the ethanol extract and 6 ml. of repurified ethylene chloride.
f. Shake well and transfer to a calibrated 15-ml. centrifuge tube.
g. Rinse the Evelyn colorimeter tube with an additional 1 ml. of ethylene chloride and add to the calibrated tube.
h. Stir and centrifuge ten minutes.
i. Remove aqueous ethanol layer with suction (total volume remaining, 7.5 ml.).
j. Pipette 2.5 ml. of the remaining 7.5 ml. of ethylene-chloride extract into a standard test tube and evaporate to dryness with air. This portion is used for determination of dehydroepiandrosterone by our micromodification of the Allen (11) method.
k. The remaining 5-ml. portion of ethylene chloride is transferred to an Evelyn colorimeter tube, the centrifuge tube being rinsed with an additional 0.5 ml. of ethylene chloride. The contents of the Evelyn tube are evaporated to dryness with an air stream. This portion is used for determination of total 17-ketosteroids by the micro-Zimmermann method.

C. Colorimetric Assay of Total 17-Ketosteroids and Dehydroepiandrosterone

Micro-Zimmermann procedure (5) for total 17-ketosteroids

a. To the dried residue (5.0-ml. portion) add:
0.2 ml. of repurified absolute alcohol.
0.2 ml. of 2.5 *N* KOH in repurified absolute alcohol.
0.2 ml. of 1 per cent metadinitrobenzene in repurified absolute alcohol.
b. Cover with foil and mix by shaking. Allow to stand ninety minutes at 25° C. in the dark.
c. Add 0.6 ml. of distilled water and 0.6 ml. of redistilled $CHCl_3$ and shake.
d. Transfer to 3-ml. test tubes and centrifuge at high speed for ten minutes, or until clear.
e. Remove water with a dropper having a long fine capillary tip.

f. Pipette the $CHCl_3$ into the microcuvettes.
g. Read in the Beckman spectrophotometer against redistilled $CHCl_3$, at 440 mμ, 520 mμ and 600 mμ. Use the Allen formula to obtain the corrected optical density.

Micro-Allen procedure for dehydroepiandrosterone

a. Add 0.2 ml. of special H_2SO_4 reagent to the residue (2.5-ml. portion).
b. Heat at 55° C. for twelve minutes.
c. Cool in cold water for one minute and add 0.3 ml. of 95 per cent ethanol.
d. Cool and pipette into microcuvette.
e. Read in the Beckman spectrophotometer at 560 mμ, 600 mμ and 640 mμ with the red phototube. A standard curve is run each time and the values are read from this curve.

Reagents and Apparatus

Reagents

1. Chloroform, freshly redistilled over K_2CO_3 on the day of use.
2. Ethanol (absolute, repurified according to Peterson (17)).
3. Methanol (distilled over 2,4 dinitrophenylhydrazine and redistilled).
4. Sulfuric acid, C.P. (Baker).
5. Ethylene chloride (Eastman Kodak) repurified as follows: Shake with 0.1 *N* NaOH (equal volume); dry with sodium sulfate, then anhydrous potassium carbonate; decant and distill.
6. Sodium hydroxide pellets, C.P.
7. Florisil (13).
8. n-Hexane, C. P.
9. Reagent for Allen determination. Special H_2SO_4 (4 parts concentrated H_2SO_4 and 1 part 90 per cent ETOH) made as follows: To 9 ml. of absolute ethanol in an Erlenmeyer flask add 1 ml. of distilled water. Immerse this mixture in ice water and add 40 ml. of concentrated H_2SO_4 drop by drop from a burette. Prepare fresh each day.
10. Reagents for Zimmermann determination—2.5 *N* KOH in absolute ethanol, and 1 per cent metadinitrobenzene in absolute ethanol.

Special apparatus

1. Shaker.
2. Beckman DU spectrophotometer.

RESULTS

Hydrolysis and extraction

Various procedures for hydrolysis and extraction of serum 17-ketosteroids have been tried in this laboratory. We successfully modified the simultaneous hydrolysis and extraction procedure of Migeon and Plager to suit our needs. This method, although much more involved than our present procedure, has been very useful and a table of normal values so obtained is presented in Table 1. During this period, Burstein and Lieberman published their studies on solvolysis techniques. We therefore decided that solvolysis offered many advantages and revised our methodology to incorporate this step.

TABLE 1. SERUM 17-KETOSTEROIDS—NORMAL VALUES BY MODIFIED MIGEON-PLAGER METHOD

Subject	Total 17-KS (Zimmermann) (μg./100 ml.)	Dehydroepiandrosterone (Allen)		Serum corticoids (μg./100 ml.)
		μg./100 ml.	% tot. 17-KS	
		Normal Females		
B.C.	75.7	47.9	63.3	—
J.C.	73.4*	46.7	63.6	—
P.F.	37.6	32.8	87.2	—
P.F.	60.8	45.3	74.5	—
J.L.	41.8*	28.7*	68.8	4.7
D.N.	86.9*	60.5*	69.6	8.3
D.N.	72.0	50.7	70.5	—
S.R.	52.9	37.4	70.7	—
Y.S.	79.4*	33.3*	42.0	11.7
P.Z.	65.2	65.7	100.0	8.3
		Normal Males		
W.B.	65.1*	37.1*	57.0	10.4
G.E.	55.8	36.0	64.5	—
R.S.	57.3*	36.3	63.4	—
H.S.	35.4	29.9	84.5	—
F.T.	33.9	0	0	13.8
J.W.	58.8*	27.0	45.8	16.5
R.Y.	74.3*	35.1	47.2	—

*Average of 2 determinations on one sample.

We now use ethyl-acetate extraction of a 2*N* sulfuric-acid solution of the diluted serum (after chloroform extraction for corticoids) to effect solvolysis of the serum 17-ketosteroid sulfates.

Removal of phenolic, acidic and pigmented material

The results obtained with sera from normal females were satisfactory

but the "method blank" values were very high. Various procedures were tried in an attempt to overcome this. Specially purified alcohol was prepared and used. The results were still not consistent enough to suit a routine procedure. We decided that part of the trouble might be eliminated if a better clean-up of the acetate extract were undertaken prior to column chromatography. If this acetate extract were to be evaporated to dryness and taken up in ethylene chloride, then the ethylene-chloride extract could be treated with pellets of sodium hydroxide in much the same manner as Drexter *et al.* (16) described in their method for determination of 17-ketosteroids in urine. This procedure was instituted and yielded lower method blank values. Its use also permitted considerable simplification of the method as follows:

a. The long washing of the ether extract with alkali and water in methods such as Gardner's is eliminated.
b. Solvent partition is carried out in a much more simple manner after column chromatography.

Column chromatography

In this step the ethylene-chloride extract is added directly to the column, followed by elution with 2 per cent methanol in chloroform. This results in improvement in time and efficiency as compared to the conventional chloroform-alcohol elution procedure.

Solvent partition

The efficiency of solvent partition for androsterone and dehydroepiandrosterone was evaluated, since losses had been noted by other workers (6, 18, 19). We found that 73 per cent of these steroids remained in the aqueous ethanol layer at this point. This correction has been applied in evaluating recoveries of these two steroids. It has not been applied to the routine determination, as steroids other than these are known to be present in serum and their distribution is not known specifically. In our procedure, the 70 per cent ethanol extract after partition with hexane is again taken up in the specially repurified ethylene chloride, since at this point the aim is to measure not only the total 17-ketosteroids but also dehydroepiandrosterone (Allen method). Our values for dehydroepiandrosterone had been erratic when we used the Allen method on the residue from the alcohol extract after solvent partition. The alcohol extract was therefore further diluted with water and re-extracted with ethylene chloride. The resultant ethylene-chloride extract was much easier to handle, in that the evaporation time for the final colorimetric assay was cut to a minimum. However, it was essential to repurify the ethylene chloride used; otherwise drastic losses of dehydroepiandrosterone occurred.

Colorimetric assay

The micro-Zimmermann procedure was used exactly as described by Migeon and Plager (5). It was observed that the ratio of optical density readings, *i.e.*, 520/420, was reasonably constant for the Zimmermann assay blank (used with standard) and for the method blank. When difficulties were encountered with high blank readings, this ratio was considerably greater than 1. The value of this ratio for the method blank based in 10 runs was $1.03 \pm .04$, and for the Zimmermann blank $1.02 \pm .03$.

The Allen method for dehydroepiandrosterone was adapted for micro amounts that were in keeping with the quantity generally found in serum. Best results were obtained with use of a calibration curve covering the range of 1 to 5 μg.

A method blank is carried through the whole procedure and is assayed by both colorimetric procedures. A 10-μg. dehydroepiandrosterone standard is carried routinely with the Zimmermann method.

It is easy to derive the equation of the line ($y=mx+b$) for each calibration curve using the method of least squares, since only y and xy change. All other terms in the equation remain as constants if the same standards and the same number of standards are carried each time. The value for y (absorbance) does change from time to time when handling such small quantities of standards; it is therefore best to run a curve for each group of samples with the Allen reaction.

Recovery of 17-ketosteroids added to serum

Recoveries of free androsterone and dehydroepiandrosterone added to serum after solvolysis are presented in Table 2. With 15-μg. amounts of added steroid, recoveries ranged from 61 to 86 per cent.

Precision

Duplicate determinations were run on a group of serum samples. The results are listed in Table 3. The maximum variations were: total 17-ketosteroids, 7.1 per cent; dehydroepiandrosterone, 15.4 per cent; and corticoids, 15.6 per cent.

Normal values

The results obtained with this method (which incorporates solvolysis) in a group of 8 normal males and 16 normal females are summarized in Table 4. The values are in the same range as those reported by others (4, 5, 10, 20).

The determinations in Table 4 were made on samples of serum obtained at irregular intervals. The age range for the females was 22 to 30 years, and for the males 25 to 50 years. No attempt was made to correlate

TABLE 2. RECOVERIES OF "FREE" 17-KETOSTEROIDS*

Pooled serum	Amt. of serum used (ml.)	μg. 17-KS added		μg. 17-KS found (Zimmermann)	% Recovery
		DHA†	Androsterone		
#1	10	0	0	11.6	—
	10	15	0	24.6	86
	10	15	0	22.8	74
#1	10	0	0	11.6	—
	10	0	15	23.1	77
	10	0	15	20.8	61
#2	10	0	0	17.5	—
	10	0	15	28.9	76

* Values corrected for 73% solvent partition factor specific for androsterone and dehydroepiandrosterone at step 5. Androsterone value corrected for 85% DHA value (Migeon) (5)).

† Dehydroepiandrosterone.

the serum steroid levels with age, physical activity, the menstrual cycle or other possible modifying physiologic factors. In the normal female, the average level of serum total 17-ketosteroids was 94.5 ± 49.8 (S.D.) μg. per 100 ml., and of dehydroepiandrosterone 50.5 ± 37.9 μg. per 100 ml. In the normal male, the average level of total 17-ketosteroids was 79.0 ± 31.3

TABLE 3. DUPLICATION OF RESULTS

Subject	Amt. of serum used (ml.)	Serum 17-KS (μg./100 ml.)		Serum corticoids (μg./100 ml.)
		Total (Zimmermann)	DHA* (Allen)	
C.M.	10.0	42.6	33.0	14.8
C.M.	10.0	41.9	28.5	14.8
D.F.	9.0	21.0	2.7	10.7
D.F.	8.5	24.2	3.3	8.5
L.E.	9.0	147.0	116.0	5.4
L.E.	9.0	161.0	115.0	7.3
C.N.	9.0	159.0	114.0	15.3
C.N.	9.0	164.0	110.0	16.8
J.L.	10.0	186.0	114.0	21.0
J.L.	10.0	196.0	114.0	27.8
J.F.	10.0	37.6	3.3	6.5
J.F.	10.0	42.4	4.5	7.2

* Dehydroepiandrosterone.

TABLE 4. NORMAL VALUES—SOLVOLYSIS PROCEDURE

Subject	Total serum 17-KS (Zimmermann) (μg./ml.)	Dehydroepiandrosterone (Allen)		Serum corticoids (μg./100 ml.)
		(μg./100 ml.)	% tot. 17-KS	
		Normal Females		
K.A.	93.6	25.5	27.3	5.5
D.N.	84.0	55.5	66.0	—
Y.S.	123.0	81.0	65.9	—
J.L.	54.0	30.0	55.5	—
B.C.	42.2	26.0	61.6	—
E.C.	83.0	34.5	41.6	—
E.C.	92.6	45.5	49.6	—
S.B.	124.0	34.7	28.0	8.9
M.M.	109.0	44.6	41.1	6.6
C.M.	42.3	30.8	72.7	14.8
D.F.	22.6	3.0	13.3	9.6
L.E.	154.0	116.0	75.5	6.3
C.N.	162.0	112.0	69.1	16.1
J.L.	191.0	114.0	59.6	24.4
J.F.	40.0	3.9	9.7	6.9
	Av. 94.5 ± 49.8	*Av. 50.5 ± 37.9*		
		Normal Males		
W.B.	88.0	15.0	17.0	—
C.S.	122.0	101.0	82.8	—
H.S.	19.9	23.4	—	—
L.F.	51.0	31.6	62.0	9.0
D.H.	82.0	27.5	33.5	10.6
G.K.	92.2	39.6	42.9	5.2
V.L.	75.8	27.4	36.1	17.3
R.B.	101.0	39.9	39.6	3.7
	Av. 79.0 ± 31.3	*Av. 38.2 ± 26.4*		

(S.D.) μg. per 100 ml., and of dehydroepiandrosterone 38.2 ± 26.4 μg. per 100 ml.

Values obtained in pathologic states are presented in Table 5 in order to indicate the applicability of the method to clinical practice.

DISCUSSION

A simple procedure has been described which permits the determination for clinical purposes of serum free 17-hydroxycorticosteroids, total 17-ketosteroids liberated by solvolysis in ethyl acetate of a 2*N* H_2SO_4 solution of serum as measured by the Zimmermann reaction, and dehydro-

TABLE 5. STEROIDS OF SERUM IN PATHOLOGIC STATES

Subject	Total serum 17-KS (Zimmermann) (μg./100 ml.)	Dehydroepiandrosterone (Allen)		Serum corticoids (μg./100 ml.)	Clinical diagnosis
		μg./100 ml.	% tot. 17-KS		
A.H.	not detectable	not detectable		9.8	Addison's disease
W.F.	100.3	66.9	64.9	16.3	Adrenal hyperplasia
H.C.	6.0	not detectable		19.1	Ménière's disease
L.B.	191.0	90.0	47.4	14.4	Adrenal hyperplasia with hypertrichosis
A.M.	11.0	10.9	100.0	16.5	Metastatic gastric carcinoma
A.P.	50.5	21.6	42.9	15.0	Menometrorrhagia; hypertrichosis
E.C.	65.9	24.3	37.0	10.8	Menometrorrhagia
E.M.	786.0	603.0	76.5	18.0	Virilism; adrenocortical tumor
A.B.	9.0	not detectable		2.9	Pituitary myxedema
K.C.	16.7	7.2	43.0	16.4	Adrenocortical tumor, postop.
M.S.	26.7	13.6	51.0	26.6	Cushing's syndrome
M.L.	37.6	19.5	51.8	8.2	Acromegaly, postop.
C.O.	114.0	66.5	63.6	17.6	Adrenal hyperplasia

epiandrosterone as measured by our micro modification of the Allen procedure (Allen blue chromogens).

Oertel and Eik-Nes (21) reported that a considerable portion of dehydroepiandrosterone in plasma can be liberated by acid or alkaline phosphatase. It is conceivable that the solvolysis procedure of Burstein and Lieberman (12) actually splits phosphate linkages as well as sulfates. Recently Purdy, Engel and Oncley (22) noted that estrone sulfate is a major estrogen constituent of plasma. Consequently, this material may well be split by ethylacetate solvolysis, and by this method a partition can be introduced between toluene and 1*N* sodium hydroxide just after evaporation of the ethyl-acetate extract at step 3b, which will allow the phenolic fraction to be set aside and processed for estrone assay.

The method devised for removal of interfering substances such as phenols and pigmented material has resulted in considerable saving of time and has increased the specificity of the determinations. For example, values for total 17-ketosteroids could be calculated directly from the 520-mμ reading to within 9 ± 6 per cent of the values obtained with the Allen formula.

The return to the use of ethylene chloride after solvent partition between hexane and alcohol increases the specificity, in that the 17-ketosteroids may exhibit more selective solubility in ethylene chloride. This was observed particularly in relation to work with dehydroepiandrosterone as determined by the Allen method.

The ratio of dehydroepiandrosterone to androsterone seems to be of the order predicted by various other workers. The micro procedure developed for the Allen color reaction, which permits calculation of this ratio, has proved to be quite satisfactory. For more accurate results, however, the quantity of extract used in the Allen assay could be increased.

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The Endocrine Society

THE 1960 ANNUAL MEETING

The Forty-Second Annual Meeting of The Endocrine Society will be held in the Eden Roc Hotel, Miami Beach, Florida, Thursday, Friday and Saturday, *June 9, 10 and 11, 1960.*

The Chairman of Local Arrangements is Dr. Kenneth Savard.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 10, at 7:30 P.M. preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Eden Roc will hold 250 bedrooms and the adjoining hotels, the Montmartre Hotel and Lucerne Hotel, will hold 75 rooms each for members until May 1, 1960, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations early, directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining through June 12 for the American Medical Association and other meetings, it is imperative that your reservation be made from June 12 on, directly through the A.M.A. Housing Bureau, c/o Miami Beach Convention Bureau, 1700 Washington Avenue, Miami Beach, Florida, since no reservation beyond June 12 can be accepted directly by the hotels. Make your reservations early and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1960.

Final program, membership card and advance registration forms will be sent on May 1, 1960 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send an original and 4 copies of the title and abstract to the Vice-President, Dr. Robert H. Williams, University of Washington School of Medicine, Seattle 5, Washington, *not later than February 15, 1960.* It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that these will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately 200 abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the

Program Committee. THE COUNCIL REQUESTS THAT AUTHORS ADHERE STRICTLY TO THE FOLLOWING RULES WHEN SUBMITTING ABSTRACTS; OTHERWISE THEY CANNOT BE CONSIDERED:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.
2. Abstracts may not exceed 200 words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formula cannot be used.
3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed 15 words;
   Line 2. Author/s. The name of each nonmember-author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of nonmembers who are introduced, *i.e.*, who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, *e.g.*, M.D., of each author should be written after his name.
   Line 3. Institution of origin and city in which institution is located.
4. Authors who wish receipt of their abstracts acknowledged should enclose self-addressed post card with the title of the abstract noted thereon.
5. Authors are also to enclose 3×5 inch filing cards, one for each author, giving his name and initials in the upper right-hand corner, and the complete title of the abstract with all the authors listed in the center of the card. There should be a second card enclosed listing the title, all authors, and the mailing address of the senior author. This is necessary for indexing. Senior authors will be notified of the acceptance of their papers for the final program.
6. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. The original and 4 copies should be forwarded to Dr. Williams.
7. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

THE 1960 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

FRED CONRAD KOCH AWARD

In 1957 a substantial legacy was bequeathed to the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, Distinguished Service Professor of Physiological Chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is the highest honor of The Endocrine Society and is represented by a medal known as the Koch Medal of The Endocrine Society. The medal, as well as an honorarium of $3,500, is to be given annually to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

This Award was first presented in 1959, to Dr. W. W. Swingle. The Koch Medal has replaced the Medal of The Endocrine Society which was established in 1954 and presented to Dr. Carl R. Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957 and Dr. I. L. Chaikoff in 1958. The Medal of the Endocrine Society replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch in 1942, (no award in 1943), Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl G. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert M. Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl Heller; 1949—Dr. George Sayers; 1950—Dr. Oscar M. Hechter; 1951—Dr. Albert Segaloff; 1952—Dr. Seymour Lieberman; 1953—Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts); 1954—Dr. Isadore M. Rosenberg; 1955—Dr. Jack Gross; 1956—

Dr. Alfred M. Bongiovanni; 1957—Dr. Nicholas S. Halmi; 1958—Dr. Monte A. Greer; 1959—Dr. Gordon L. Farrell. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

THE AYERST AND THE SQUIBB FELLOWSHIPS

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

THE UPJOHN SCHOLAR OF THE ENDOCRINE SOCIETY

The Council of The Endocrine Society has established a category of Scholars. A grant has been made available through the generosity of the Upjohn Company, and will be awarded to an established investigator or teacher in the field of endocrinology who wishes to extend the opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually and will be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award, the Ciba Award, and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be

obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *October 15* each year.

Proposals for appointments as a Scholar of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *October 15* each year.

ELEVENTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM

SPONSORED JOINTLY BY THE ENDOCRINE SOCIETY AND THE UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE AND DEPARTMENT OF CONTINUING MEDICAL EDUCATION

SAN FRANCISCO, CALIFORNIA

November 9 through 13, 1959

For information concerning the program and registration, write to *Seymour M. Farber, M.D.*, Assistant Dean in charge of Continuing Medical Education, University of California Medical Center, San Francisco 22, California.

The registration fee is $100 payable to the Regents of the University of California. Residents and Fellows will be admitted for a reduced fee of $30. Those wishing to reserve rooms should communicate directly with the Whitcomb Motor Hotel, 8th and Market St., San Francisco.

(For program, see the June issue of the Journal)

FIRST INTERNATIONAL CONGRESS OF ENDOCRINOLOGY

The First International Congress of Endocrinology will be held in *Copenhagen, Denmark, July 18–23, 1960.*

United States members of the International Executive Committee are Dr. Warren O. Nelson and Dr. Leo T. Samuels, Dr. Gregory Pincus is Chairman of the Program Committee. The tentative program consists of ten symposia covering the general field of endocrinology plus round-table discussions and a number of submitted papers. Those desiring to submit papers should immediately contact Dr. Gregory Pincus, Worcester Foundation, Shrewsbury, Massachusetts. There will be a number of interesting scientific and technical exhibits.

In order to facilitate travel arrangements to the Copenhagen Convention, the Council of The Endocrine Society at its San Francisco meeting designated THOMAS COOK & SON as our authorized travel agents. They will in turn offer especially designed All Expense Paid Tours to Europe, including hotel reservations for the convention, as well as offering independent itineraries for any of our members who desire to travel separately by air or by steamship.

Registration is open to anyone interested in endocrinology at an approximate fee of $28 U.S. Members of the families of those attending may register as associates at an approximate fee of $14 U.S., fee to be paid at the time of registration or in advance to the American Express Company, which has been designated by the Executive Committee of the International Congress as official financial and travel agent. There will be a number of social functions and other entertainment provided for all registrants.

As you all know, European travel is unusually heavy during July and it is suggested that those contemplating attending the Congress should start making tentative reservations at the earliest possible moment by filling in and returning the card recently mailed to members, to THOMAS COOK & SON's special representative—Harry E. Kornbaum, Transportation Chairman, Rainbow Travel Service, 2817 Classen Boulevard, Oklahoma City 6, Oklahoma, who will correspond directly with each member about his individual travel arrangements to and from the Convention, at the same time keeping our National Officers fully informed of all travel arrangements of its members. Completion of the card CARRIES NO OBLIGATION OR COMMITMENT ON YOUR PART. Send it in today for the complete travel arrangements for our Society.

The general expenses of a meeting of this magnitude are quite large and The Endocrine Society has made a substantial contribution from its treas-

ury for organizational purposes. U.S. pharmaceutical firms have also contributed very generously. However, sufficient funds are not as yet available to assure the success of this meeting; therefore, *the committee suggests that each of our members make voluntary contributions to this fund.* Please make your checks payable to: The Endocrine Society—First International Congress of Endocrinology. Mark it *Contribution* and mail it to the office of the Secretary-Treasurer, 1200 North Walker Street, Oklahoma City 3, Oklahoma.

PROGRAM

The program will consist of ten Symposia and a Round Table Discussion, and of groups of shorter (ten-minute) communicated papers. Speakers at the Symposia and the Round Table Discussion are invited by the Congress. Communicated papers may be submitted by investigators in the field of endocrinology, and titles and abstracts are invited. Forms for registration in the Congress, along with forms for the submission of abstracts of short communications, may be obtained by writing to the Congress Secretariat: Dr. Svend G. Johnsen, Hormone Department, Statens Seruminstitut, Copenhagen S, Denmark. The final date for the submission of such abstracts is *December 31, 1959.* The official languages of the Congress are English, French, German and Spanish. A volume containing advance abstracts of symposium contributions and the short communications (in one of the official languages and translated into Interlingua) will be distributed to members on registration at the Technical University of Denmark in Copenhagen. Titles and abstracts of communicated papers should not be sent to the Program Committee, but the Program Committee will review and arrange for the presentation of such papers in appropriate Congress sessions.

GREGORY PINCUS, *Chairman,*
Subcommittee on Program

The American Goiter Association

THE 1960 ANNUAL MEETING

The next Annual Meeting of the American Goiter Association will be held in *London, England, July 5–9, 1960,* in the Great Hall of the Royal College of Surgeons. This meeting, co-sponsored with the London Thyroid Club, will be the Fourth International Goiter Conference. For further information write Dr. Selwyn Taylor, 3 Roedean Crescent, Roehampton, London, S.W.15, England.

JOHN C. MCCLINTOCK, *Secretary,*
149½ Washington Avenue,
Albany 10, N.Y.

SUBMISSION OF ABSTRACTS FOR THE PROGRAM OF THE FOURTH INTERNATIONAL GOITER CONFERENCE

Abstracts of American papers to be considered for presentation at the Fourth International Goiter Conference, London, England, July 5–9, 1960, should be sent to Dr. J. E. Rall, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda 14, Maryland. Abstracts from all other countries are to be sent to Dr. Selwyn Taylor, 3 Roedean Crescent, Roehampton, London, S.W.15, England.

All American abstracts must be received by Dr. Rall *by December 1, 1959.* They should not exceed 400 words and should be submitted in quintuplicate. Presentation of papers will be limited to fifteen minutes and a copy of the final paper must be sent to the London Secretary two weeks prior to the meeting.

THE VAN METER PRIZE AWARD FOR 1960

The American Goiter Association again offers the Van Meter Prize Award of $300.00 to the essayist submitting the best manuscript of original and unpublished work concerning "Goiter—especially its basic cause." The studies so submitted may relate to any aspect of the thyroid gland in all of its functions in health and disease. The Award will be made at the Fourth International Goiter Conference in London, England, July 5–9, 1960, where a place on the program will be reserved for the winning essay-

ist if he can attend the meeting. For 1960, the recipient of the Award will receive consideration for an award of a travel honorarium.

The competing essays may cover either clinical or research investigations, should not exceed 3,000 words in length and must be presented in English. Duplicate typewritten copies, double spaced, should be sent to the Secretary, Dr. John C. McClintock, 149½ Washington Avenue, Albany 10, New York, *not later than January 1, 1960.* The committee who will review the manuscripts is composed of men well qualified to judge the merits of the competing essays.

FOUNDING OF THE INTERNATIONAL SOCIETY OF CYBERNETIC MEDICINE

The International Society of Cybernetic Medicine was recently founded with the purpose of assembling researchers engaged in the study of Cybernetics applied to Medicine and Biology, and diffusing this method of research based upon the analogic calculus and the use of electronic models. Physicians, biologists, engineers, physicists and mathematicians may become members of this Society. Applications should be addressed to the Secretariat-General, presently located at 348 Via Roma, Naples, Italy. The Society by-laws will be sent upon request.

NUCLEAR-MEDIZIN

"Nuclear-Medizin," first published in 1959, covers the rapidly growing field of artificial radioactive isotopes in medical research, therapy and diagnosis. In the first issue approximately half of the articles concern the use of radioisotopes in endocrine disorders. The journal will appear four times a year. Subscription price, 11 DM. Friedrich-Karl Schattauer-Verlag, Stuttgart 1, West Germany.

NEW YORK ACADEMY OF MEDICINE THIRD ANNUAL POSTGRADUATE WEEK

The program, entitled "Research Contributions to Clinical Practice," will be conducted *October 5 through October 9, 1959,* and will consist of daily evening lectures, daily afternoon panels, and a scientific exhibit. Although registration is required of non-Fellows of the Academy, no fee will be charged. All inquiries should be addressed to Secretary, Postgraduate Week, The New York Academy of Medicine, 2 East 103 Street, New York 29, N. Y.

THE ACADEMY OF PSYCHOSOMATIC MEDICINE

The sixth annual meeting of The Academy of Psychosomatic Medicine will be held *October 15–17, 1959* at the Sheraton-Cleveland Hotel in Cleveland. The meeting will be oriented and directed to fit the needs of non-psychiatric physicians. Practical everyday office management of psychosomatic problems and emotional disturbances will be dealt with in formal papers, symposia, panel discussions, and small study groups.

The meeting will be open to all scientific disciplines, as well as psychologists, social workers and nurses. Information may be obtained from Dr. Bertram B. Moss, Suite 1035, 55 East Washington Street, Chicago 2, Illinois.

BOOK REGISTER

Proceedings of the Second World Congress on Fertility and Sterility. Vols. I and II. Edited by G. TESAURO, Professor of Obstetrics and Gynecology, University of Naples, Italy, for the International Fertility Association, with the aid of a grant from the Government of the Italian Republic. Opening Address by His Holiness, POPE PIUS XII. In Volume I the papers deal with: Endocrine, metabolic and hematological factors in fertility and sterility, endocrine therapy in female sterility, diagnosis of ovulation and its disorders, treatment of disorders of ovulation, tubal physiology and its disorders, and evaluation of new methods of diagnosis and medical treatment of female sterility; 1390 pages, illustrated. In Volume II the papers deal with: Occupational, toxic and psychologic factors in fertility and sterility, surgery in the treatment of female sterility, experimental and clinical investigations in female sterility, diagnosis of spermatogenesis and its disorders, treatment of disorders of spermatogenesis, new methods of diagnosis of male sterility, surgery in male sterility, problems in animal reproduction, motion pictures, scientific exhibits, and indexes; 1560 pages; illustrated. The articles are in several languages, but many are in English. Institute of Clinical Obstetrics and Gynecology, University of Naples, Naples, Italy.

Regulation of Cell Metabolism. Ciba Foundation Symposium, edited by G. W. E. WOLSTENHOLME, M.B., B.Ch. and C. M. O'CONNOR, B.Sc. Thirty-nine internationally known authorities present a pioneer symposium on: Rate-limiting factors in cell respiration, the meaning of intracellular structure for metabolic regulation, topographical aspects of the regulation of cell metabolism, quantitative aspects of control of oxygen utilization, control points in phosphorylating respiration, choice of oxidative pathways of carbohydrate metabolism, the mechanism of glycogen synthesis in muscle, alternative pathways of electron transport, limiting factors in glycolysis of ascites tumor cells, role of triphosphopyridine nucleotide in the regulation of glycolysis in a cell-free preparation, phosphate turnover and Pasteur effect, enzymic regulation of fermentation in yeast cells, mechanisms for control of enzyme synthesis and activity in bacteria, automatic adjustment mechanisms in bacterial cells, and regulation of growth and composition of the bacterial cell; 388 pages; 109 illustrations; 1959. Little. Brown and Company, Boston, Mass. Price $9.50.

Transactions of the Josiah Macy, Jr. Foundation Conferences. Publications Office, 16 West 46th Street, New York 36, N. Y.

Neuropharmacology. Fourth Conference, September 25–27, 1957, Princeton, N. J. Edited by HAROLD A. ABRAMSON, M.D., Research Psychiatrist, The Biological Laboratory, Cold Spring Harbor; Consultant in Research Psychiatry, State Hospital, Central Islip, New York. The 4 sections deal with the following subjects: The effect of respiratory poisons and anoxia on Siamese fighting fish in relation to LSD-25 reaction, clinical studies with taraxein, "stop" and "start" mechanisms, and some relations between chemical structure and physiologic action of mescaline and related compounds; 286 pages; 48 figures; 12 tables; 1959. Price, $5.00.

Physiology of Prematurity. Third Conference, March 25–27, 1958, Princeton, N. J. Edited by JONATHAN T. LANMAN, M.D., Department of Pediatrics, New York University-Bellevue Medical Center, New York, N. Y. The subjects covered are: Metabolic and enzymic changes during development, bile pigment metabolism and liver function in premature infants, congenitally jaundiced rats, glucuronic acid in the treatment of bilirubinemia, and biochemical mechanisms in glucuronide formation; 158 pages; 56 figures; 9 tables; 1959. Price, $3.00.

Tuberculosis Medical Research. The National Tuberculosis Association, 1904–1955. Historical Series No. 9. by VIRGINIA CAMERON, formerly Medical Research Secretary and ESMOND R. LONG, M.D., formerly Director of Medical Research, National Tuberculosis Association. This book reviews the development of a medical research program supported by voluntary contributions. It offers a scientific and historical perspective that other organizations planning research programs may find valuable; 325 pages; illustrated; 1959. The National Tuberculosis Association, 1790 Broadway, New York 19, N. Y. Price, $5.00.